Proceedings
of the
Society
for
Experimental Biology and Medicine

OLUME 61 FEBRUARY, 1946 NUMBER 2

SECTION MEETINGS

ACIFIC COAST
University of California Medical School December 12, 1945

15238 P

Sodium Salicylate Inhibiting Anti-Rh Immunization in Animals.

F. HOMBURGER. (Introduced by G. R. Minot.)

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston.

The inhibition of anti-RH agglutinins by hylmercuri-thiosalicylate *in vitro* has been own by Waller[1] and sodium salicylate is own to impair the formation of antipneu- ococcic immune antibodies in rabbits.[2] The esent preliminary study suggests that sodi- n salicylate inhibits the formation of anti- h agglutinins in guinea pigs and in rabbits.

Experimental Procedure. Forty guinea gs and 20 rabbits were used. All ani- als were immunized by the injection of d blood cells from rhesus monkeys. Guinea gs received intraperitoneal injections of cc of washed rhesus cells suspended in line on the third and on the eighth day the experiment. Rabbits were given in- avenous injections of 0.01 cc of citrated esus blood in 1 cc of saline on the 4th, h, 6th, 11th, 12th and 13th days of the periment. Half of the number of the ani- als received 0.16 g of sodium salicylate r kg of body weight daily by stomach tube uinea pigs) or by subcutaneous injection abbits) starting the first day of the ex- riment and continued up to 48 hours before the blood was examined, 7 days after the last injection of rhesus blood. Anti-Rh agglutinins were looked for by the method of Landsteiner and Wiener[3] in the case of the guinea pigs* and by a modification of Diamond's[4] method, using human albumin† in the case of the rabbits. Negative readings were confirmed by microscopic examination. Serum salicylate levels were measured by the method used by Coburn.[5]

Results and Discussion. Of 21 animals‡ that had not received any sodium salicylate, 15 showed anti-Rh agglutinins following the injection of rhesus blood. In the case of the rabbits these were stronger which suggests that the methods used on these animals were better suited for such experiments.

---

[1] Waller, K., *Am. J. Clin. Path.*, 1944, **8**, 116.

[2] Swift, H. F., *J. Exp. Med.*, 1922, **36**, 735.

[3] Landsteiner, K., and Wiener, A. S., *J. Exp. Med.*, 1941, **74**, 309.

[4] Diamond, L. K., and Denton, R. L., *J. Lab. and Clin. Med.*, 1945, **10**, 821.

* Some of these sera were examined by Dr. L. K. Diamond whose cooperation is gratefully acknowledged.

† The albumin used was a sample prepared by the Harvard Fractionation Laboratory and obtained through the courtesy of Prof. E. J. Cohn.

[5] Coburn, A. F., *Bull. Johns Hopkins Hosp.*, 1943, **73**, 435.

‡ An epizootic described elsewhere[6] destroyed 9 of the guinea pigs and 12 rabbits died from aspiration pneumonia.

[6] Homburger, F., Wilcox, C., Barnes, M. W., and Finland, M., *Science*, 1945, **102**, 449.

Of 18 animals which had received sodium salicylate during the period of immunization only 2 showed microscopic agglutination of Rh positive cells.

No free salicyl radical was found in any of the sera tested which suggests that *in vivo* salicylate inhibits the formation of agglutinins rather than their effect in the plasma.

These findings call for confirmation and salicylate prophylaxis for erythroblastosis fetalis is not suggested here. On the contrary, the toxicity of this drug is again[7] emphasized. However, these experiments intimate the possibility of a chemotherapeutic approach to the subject of iso-immunization.

Summary. In guinea pigs and in rabbits the formation of anti-Rh agglutinins following the injection of rhesus monkey blood cells appears to be diminished when sodium salicylate has been administered 3 days prior to and during the period of immunization.

---

[7] Homburger, F., *Am. J. Med. Sci.*, in press.

15239 P

Biochemical Changes Following Poisoning of Rats by Alpha-naphthylthiourea.

K. P. DuBois, L. W. Holm, and W. L. Doyle.
*From the University of Chicago Toxicity Laboratory.**

Alphanaphthylthiourea (ANTU) has been proposed as a rodenticide because of its effectiveness against Norway rats. Richter[1] has shown that poisoning by this compound is characterized by (a) development of intense pulmonary edema and pleural effusion, (b) marked resistance of herbivores in comparison to carnivores, (c) greater resistance of younger animals, (d) a rapid development of tolerance after repeated sublethal doses. These factors are individually significant in the action of many drugs. The present study indicates that ANTU profoundly alters the carbohydrate metabolism in poisoned animals and that the administration of cysteine counteracts these effects in rats.

Experimental. Purina Chow was used as diet for adult albino rats. ANTU was administered intraperitoneally in propylene glycol (5 mg ANTU per ml solvent). The solvent alone produced no observable effects. Under these conditions the LD-50 was approximately 3.0 mg per kilo. A dose of 10 mg per kg killed all rats between 6 and 24 hours after injection. Glucose was determined by the method of Folin and Malmros[2] and glycogen according to Good *et al.*[3] Our initial experiments consisted blood glucose determinations on rats prior to and 2.5 hours after injection of 10 mg per kg of ANTU. In 6 rats the average normal blood glucose rose from 68 ± 2 mg % to an average of 173 ± 18 mg % in 2.5 hours after poisoning. It was later observed that this initial hyperglycemia was followed by hypoglycemia which might be due to depletion of liver glycogen. Eight rats were fasted for 7 hours after which time 4 received 10 mg per kg of ANTU intraperitoneally. Food was withheld from both groups for another 8 hours after which

---

* This work was carried out under contract with the Medical Division of the Chemical Warfare Service.

[1] Richter, C. P., *J. Am. Med. Assn.*, 1945, **129**, 927.

[2] Folin, O., and Malmros, H., *J. Biol. Chem.*, 1929, **83**, 115.

[3] Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 484.

TABLE I.
Effect of ANTU on Synthesis of Liver Glycogen.

Glucose schedule	Controls		ANTU—10 mg/kg	
	Rat No.	Glycogen %	Rat No.	Glycogen %
None	15	0.14	22	.11
	16	0.14	23	.07
0.15 g/hr for 2 hr before death	17	0.48	24	.09
	18	0.28	25	.12
” ” ” 4 ” ” ”	19	0.95	26	.03
	20	1.20		
” ” ” 6 ” ” ”	21	1.20	27	.10
			28	.05

TABLE II.
Effect of Cysteine on the Toxicity of ANTU.

Group	ANTU (mg/kg)	Cysteine (mg/kg)	Mortality	% Mortality
1	10	0	5/5	100
2	10	200	2/2	100
3	10	400	3/5	60
4	10	700	1/5	20
5	10	1000	0/5	0

time all of the animals were sacrificed by decapitation. The livers were removed and frozen on dry ice. Decapitation and removal of the livers was accomplished within 1 minute to prevent glycogenolysis. Whereas the average liver glycogen values for the control animals was 1.13% ± .25, that of the ANTU-poisoned group was only 0.019% ± .006, clearly indicating a depletion of liver glycogen in rats poisoned with ANTU.

The glycogenic activity of the liver of ANTU-poisoned animals was then examined. Fourteen female rats were fasted 16 hours after which time 7 rats were given 10 mg per kg of ANTU intraperitoneally. A 10% glucose solution was injected intraperitoneally at hourly intervals into the fasted controls and the ANTU-poisoned animals. All the animals were killed 6 hours after ANTU was injected and liver glycogen was determined as previously described. The quantity of glucose injected per rat and the results of the liver glycogen determinations are given in Table I. The animals which had received 10 mg per kg of ANTU were unable to deposit liver glycogen, while controls deposited glycogen after glucose injections.

Possible methods of counteracting ANTU poisoning are of interest not only with respect to the mechanism of action but also from the standpoint of obtaining therapeutic measures for accidental poisoning. It appeared possible that ANTU reacts with sulphydryl groups. Cysteine was the first sulphydryl compound tested. Rats were given 10 mg per kg of ANTU (5 mg per ml in propylene glycol) intraperitoneally. Varying amounts of cysteine (100 mg per ml in saline) were injected intraperitoneally into 5 groups of rats immediately after injection of ANTU. The results given in Table II show that cysteine, at a level of 1 g per kg effectively counteracted the lethal action of an LD-100 dose of ANTU, and partial protection was obtained by lower doses of cysteine in rats. Additional results indicate that intraperitoneal injection of cysteine is equally effective against ANTU administered by stomach tube. Protection to the same degree was not obtained in dogs receiving a lethal dose of ANTU.

Summary and Conclusions. The intraperitoneal injection of 10 mg per kg of ANTU into rats caused a marked rise in blood sugar from an average value of 68 mg % and 173 mg % in 2.5 hours. Liver glycogen values fell from a normal of 1.13% to 0.019%. ANTU-poisoned animals were unable to deposit liver glycogen. While these experiments demonstrate a disturbance in carbohydrate metabolism, they do not prove that death from ANTU is due to these changes. In rats large doses of cysteine (1000 mg per kg) afforded protection from death against 10 mg per kg of ANTU. The relation between lung damage and impairment of glycolysis and/or respiration is under investigation.

15240

Modification of Anaphylaxis by Benadryl.*

J. A. Wells, H. C. Morris, and Carl A. Dragstedt.

From the Department of Pharmacology, Northwestern University Medical School, Chicago, Ill.

It has been recently shown that beta-dimethyl aminoethyl benzhydryl ether hydrochloride (Benadryl) inhibits the action of histamine on the bronchi and ileum of the guinea pig.[1] The subsequent demonstration that this drug is capable of reducing the severity of anaphylaxis in the guinea pig[2] is in accordance with the repeated demonstration that anaphylaxis in the guinea pig is due, in large part, to bronchospasm resulting from the action of histamine which is liberated from the lung.[3–8]

We have demonstrated that Benadryl inhibits the vasodepressor action of histamine in the anesthetized dog and have suggested that this inhibition is due to competition of Benadryl with histamine for its site of action.[9,10] The purpose of the present investigation was to determine the effect of Benadryl against anaphylaxis in the dog, a condition which, as in the guinea pig, is intimately connected with the release and action of histamine.[11–15]

It has been shown that in fatal anaphylaxis in the dog from 3.2 to 4.7 mg of histamine per kilo (measured as acid phosphate) is liberated from the liver.[16] It is also known that such doses of histamine injected intravenously in dogs produce fatal shocks. [13,17] Therefore, preliminary to studies of anaphylaxis, a series of 8 dogs were injected intravenously with 4.5 mg of histamine acid phosphate per kilo. These animals were pretreated with a dose of Benadryl (10.0 mg

---

* This study was aided in part by a grant from the Clara A. Abbott Fund of Northwestern University Medical School.

The Benadryl used in these studies was furnished to us through the courtesy of Parke, Davis and Company.

[1] Loew, E. R., Kaiser, M. E., and Moore, V., *J. Pharm. and Exp. Therap.*, 1945, **83**, 120.

[2] Loew, E. R., and Kaiser, M. E., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 235.

[3] Bartosch, R., Feldberg, W., and Nagle, E., *Pflüger's Arch.*, 1932, **230**, 129.

[4] Wachstein, M., *Pflüger's Arch.*, 1932, **231**, 24.

[5] Daly, I., Peat, S., and Schild, H., *Quart. J. Exp. Physiol.*, 1935, **25**, 32.

[6] Ungar, G., and Parrott, J. L., *C. R. Soc. de biol.*, 1936, **123**, 676.

[7] Ungar, G., Parrott, J. L., and Levillain, A., *C. R. Soc. de biol.*, 1937, **125**, 1015.

[8] Schild, H. O., *J. Physiol.*, 1937, **90**, 34P.

[9] Wells, J. A., and Morris, H. C., *Fed. Proc.*, 1945, **4**, 140.

[10] Wells, J. A., Morris, H. C., Dragstedt, Carl A., and Bull, H. B., *J. Pharm. and Exp. Therap.*, in press.

[11] Dragstedt, C. A., and Gebauer-Fuelnegg, E., *Am. J. Physiol.*, 1932, **102**, 512.

[12] Gebauer-Fuelnegg, E., and Dragstedt, C. A., *Am. J. Physiol.*, 1932, **102**, 520.

[13] Dragstedt, C. A., and Mead, F. B., *J. Pharm. and Exp. Therap.*, 1936, **57**, 419.

[14] Code, C. F., *Am. J. Physiol.*, 1939, **127**, 78.

[15] Dragstedt, C. A., and Mead, F. B., *J. Immunol.*, 1936, **30**, 319.

[16] Ojers, G., Holmes, C. A., and Dragstedt, C. A., *J. Pharm. and Exp. Therap.*, 1941, **73**, 33.

[17] Dragstedt, C. A., and Mead, F. B., Proc. Soc. Exp. Biol. and Med., 1935, **32**, 1435.

TABLE I
Influence of Benadryl on Anaphylaxis in the Dog.

Degree of shock	No. and % of animals manifesting various degrees of shock			
	(26) Non-treated controls		(22) Benadryl (10 mg/kilo)	
	No.	%	No.	%
0	8	30.8	3	13.6
+	2	7.7	4	18.2
++	7	26.9	13	59.1
+++	0	0.0	2	9.0
++++	9	34.6	0	0.0

per kilo i. v.) which previous experience has shown to produce nearly maximum histamine antagonism. All of these animals had severe shocks, but none of them died. This experiment suggests that if histamine is the primary toxic factor in anaphylaxis, then no animals pretreated with 10.0 mg per kilo of Benadryl should die from anaphylaxis.

Forty-eight dogs were sensitized to horse serum by injecting 5 cc intravenously and 5.0 cc subcutaneously. From 13 to 15 days later these animals were anesthetized with sodium barbital and prepared for recording the carotid blood pressure. All animals were given a shocking dose of 10 cc of horse serum intravenously. In 22 of these animals, prior to the injection of horse serum, Benadryl (10 mg per kilo) was slowly injected intravenously.

The fall in blood pressure occurring as a consequence of anaphylaxis was roughly quantitated as follows: 0 = no fall in blood pressure, + = fall in blood pressure, but not to shock level (*i.e.* 35-45 mm. of mercury), ++ = fall in blood pressure to shock level, but with recovery beginning within 30 min., +++ = fall in blood pressure to shock level with no recovery within 30 min., ++++ = fall in blood pressure to shock level with death within 30 min.

Comparison of the 2 groups of animals (Table I) reveals that in contrast to a 34.6% mortality in the untreated series, no dogs died of anaphylaxis when they had been previously treated with 10 mg per kilo of Benadryl. It is apparent from the table that all evidence of anaphylactic vasodepression is not removed by Bernadryl. This is not surprising since we have shown that maximally effective doses of this drug do not completely abolish the actions of injected histamine in the dog. Therefore, assuming histamine to be the sole toxic factor in anaphylaxis in the dog, Benadryl would not be expected to completely abolish anaphylaxis.

It is not inconceivable that Benadryl modifies anaphylaxis in the dog by some action other than an antagonism of the histamine which is released. One obvious possibility is that it prevents the release of histamine. This possibility was investigated in the following way. Two dogs which had been previously sensitized to horse serum were shocked following the administration of 3.0 mg/kilo of Benadryl. At the point at which their respective blood pressures reached shock level a sample of their blood was drawn, heparinized and centrifuged. The serum from these dogs was then assayed by comparison with standard solutions of histamine acid phosphate on the blood pressure of the atropinized anesthetized cat. The outcome of the shock in the two animals was +++ and ++ respectively. The dog showing +++ shock was shown to have the equivalent of over 10 mcgm of histamine acid phosphate per cc of serum. The dog having ++ shock was shown to have the equivalent of 1 mcgm of histamine acid phosphate per cc of serum. These are amounts of histamine which are encountered in substantial anaphylactic shocks in the absence of Benadryl and indicate that Benadryl does not materially interfere with the release of histamine in anaphylactic shock in the dog.

Summary. The intravenous injection of Benadryl (10 mg per kilo) into horse serum sensitized dogs, prior to the reinjection of horse serum, reduces the severity of anaphylactic shock in these animals, there being no deaths in 22 animals as against 9 deaths in 26 controls. As Benadryl has a similar modifying effect upon the shock induced by the injection of histamine, the results of these experiments are consistent with the theory that histamine plays a significant role in anaphylaxis in dogs. Unfortunately, Benadryl merely reduces, but does not obliterate, the vasodepressor effects of histamine and thus the present experiments with Benadryl do not permit conclusions as to whether histamine is or is not the sole vasodepressor factor in anaphylaxis in the dog.

15241

Corneal Reaction to Viruses of Equine Encephalomyelitis After Intra-Ocular Injection.*

C. A. EVANS AND V. S. BOLIN.

From the Department of Bacteriology and Immunology, School of Medicine, University of Minnesota.

In a previous publication[1] it was reported that injection of certain viruses into the eyes of suitable animals caused specific reactions in the ocular tissues. Corneal edema and opacity were produced when the viruses of fox encephalitis, influenza, and equine encephalomyelitis were employed. It was demonstrated that virus of fox encephalitis, even in minute dosage, would infect the corneal endothelium and induce the corneal reaction in dogs, foxes and raccoons.[2,3] The influenza virus caused an entirely similar reaction in the eyes of rabbits in spite of a complete absence of infection, by a direct toxic effect.[4] The virus did not grow in the rabbit eye and was effective only in very large doses.

Further studies reported in this paper suggest that the corneal opacity in rabbits caused by the viruses of western and eastern equine encephalomyelitis is analogous to the toxic reaction demonstrated for influenza virus even though these viruses may grow to a limited extent in the ocular tissues.

In our first experiment a 10^{-3} dilution of mouse brain infected with a highly virulent strain of virus of W.E.E. (Western Equine Encephalomyelitis) was injected into the anterior chamber of one eye of each of 3 rabbits. At 48 hours all showed some cloudiness of the cornea and at 3 days the corneas of all injected eyes were opaque.

In subsequent experiments 27 rabbits have been injected with 2 different strains of western virus. One of the western strains was highly virulent and had been maintained by passage in mice for many years by Dr. Carl M. Eklund, who obtained it from Dr. P. K. Olitsky of the Rockefeller Institute for Medical Research. This we call the Rockefeller strain. The second strain of western virus had been isolated from a fatal human case of encephalitis by injection of brain tissue into guinea pigs. Some of the infected guinea pig brain tissue that had been kept frozen for more than a year was used to infect guinea pigs and mice, the brains of which served as the source of virus for injection into eyes. This is called the "low passage" strain of virus.

---

* Aided by grants from the John and Mary R. Markle Foundation and the Graduate School of the University of Minnesota.

[1] Evans, C. A., Yanamura, H. Y., and Green, R. G., *Science*, 1943, **98**, 45.

[2] Evans, C. A., Yanamura, H. Y., and Green, R. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 183.

[3] Green, R. G., Evans, C. A., and Yanamura, H. Y., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 186.

[4] Evans, C. A., and Rickard, E. R., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 73.

TABLE I.

Tests for Virus in Aqueous Humor of Rabbit Eye. All animals were injected with 0.2 ml of a 10% suspension of mouse brain infected with the Rockefeller strain of western equine encephalomyelitis virus.

				Fate of mice injected with dilutions			
	Rabbit No.	Eye	Interval*	10^{-0}	10^{-1}	10^{-2}	Cornea
Exp. No. I	K423	Rt.	60 hr	†5 5 s	s s s	s s s	Opaque
		Left	,, ,,	s s s	s s s	s s s	,,
	K424	Rt.	,, ,,	s s s	s s s	s s s	Slight cloudiness
		L.	,, ,,	s s s	s s s	s s s	,, ,,
II	K433	Rt.	24 hr	2 2 2	2 2 2	2 2 2	Insufficient time
		L.	,, ,,	2 2 2	2 2 2	2 2 2	,, ,,
	K435	Rt.	3 days	s s s	s s s	s s s	Clear
		L.	,, ,,	s s s	s s s	s s s	,,
III	K425	Rt.	5 ,,	s s s	s s s	—	,,
		L.	,, ,,	s s s	s s s	—	,,
	K426	Rt.	,, ,,	3 3 3	3 3 3	—	Opaque
		L.	,, ,,	s s s	s s s	—	,,
IV	K501	Rt.	3 days	s s s	s s s	s s s	,,
		L.	,, ,,	s s s	s s s	s s s	,,
	K503	Rt.	2 ,,	5 5 s	5 s s	s s s	,,
		L.	,, ,,	3 3 3	3 3 4	4 4 4	,,

* Time between inoculation into ant. chamber and aspiration of aqueous humor.

† Numbers = day of death. S = survived.

Injection of the 12 eyes of 6 rabbits with various dilutions of the low passage strain of virus gave entirely negative results. Five of the eyes were injected with a 10% suspension of infected brain. All eyes, except 2, were injected with an inoculum which killed mice regularly in a dilution of 10^{-7} and sometimes in dilutions of 10^{-8}.

In further studies with the Rockefeller strain, negative results have been obtained in all eyes injected with virus (infected mouse brain) diluted to 1:100 or beyond. When a 10% suspension was employed, only half of the infected eyes showed the characteristic reaction. This was surprising in view of the results of the first experiment in which a 10^{-3} dilution was injected and all 3 injected eyes were positive. Unfortunately, the records showing the results of the titration of the virus used in this first experiment were lost. The later experiments were carried out after an interval of more than one year with virus that killed all mice injected with a 10^{-6} and about half those given 10^{-7} dilution. It was our recollection that the virus used in the earlier experiment killed mice in a dilution of 10^{-8}.

As shown in Table I Experiments I and III, rabbits injected in the same experiment with the same dose of virus might differ in their reactions. Numerous aspirations and cultures of aqueous humor showed an absence of pus cells and bacteria. It was felt that the varying results indicated that the amount of virus injected approximated one 50% minimal reactive dose (analogous to "1 MLD 50").

Tests for virus in aqueous humor were carried out at 24 hours, 48 hours, 60 hours, 3 days, and 5 days with the results shown in Table I. After injection of more than 300,000 mld (for mice) of virus into the anterior chamber, the aqueous humor contained virus in considerable amount at 24 hours but little or none remained at 48 hours and later. No virus was detected in ocular tissues at 3 days in 1 experiment. Of 4 eyes tested at 5 days, one contained a moderate amount of virus. This suggests that some growth of virus may have occurred. Of the 30 rabbits used in these experiments, only 3 showed symptoms indicative of encephalitis.

An eastern strain of equine encephalomyelitis virus obtained from Dr. Carl M. Eklund has been injected into the 10 eyes of 5 rabbits. All 10 eyes developed the

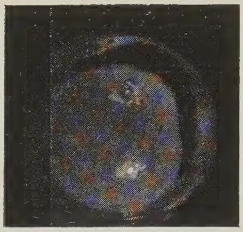


FIG. 1.

Rabbit eye 4 days after injection of the virus of eastern equine encephalomyelitis into the anterior chamber (K513).

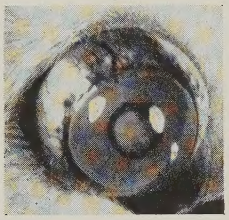


FIG. 2.

Rabbit eye showing minimal cloudiness of cornea with a typical hyperemia of subconjunctival vessels associated with uveitis. K424, 3 days after injection of western equine encephalomyelitis virus.

characteristic corneal opacity, usually of severe degree. The inoculum in each case was a 10% suspension of infected mouse brain. Titrations of the inocula by intracerebral injection in mice have shown that a 10^{-7} dilution was regularly fatal and a 10^{-8} dilution occasionally killed mice.

Tests for virus in the aqueous humor and/or ocular tissues have been made at 2, 4 and 11 days. Results as presented in Table II show that a negligible amount of virus remained. This was true even in an animal suffering from a severe encephalitic infection.

Of the 5 rabbits injected with the eastern virus, 2 were killed at 2 and 4 days and 1 of the remaining 3 developed encephalitis.

Discussion. From the data presented in Tables I and II, it is clear that the corneal opacity observed in rabbits after intraocular injection of the viruses of western equine encephalomyelitis is not the result of the growth of virus in the eye, even though such growth may occur to a limited extent. In control experiments the injection of suspensions of normal brain and of numerous other non-viral preparations into rabbits' eyes has never in our experience caused the type of ocular reaction that characterized the experiments with these viruses or influenza viruses. As in the experiments with influenza virus, large doses of equine virus (in the vicinity of a million mld for mice) must be injected in order to produce the corneal reaction. In most of our experiments, the dosage of western virus was apparently close to the minimum level necessary to produce a reaction inasmuch as frequently some eyes were negative while others were positive. The minimum dose of eastern virus that will cause the reaction was not determined.

In the present paper, attention is centered on the corneal reaction because this is the most prominent and most characteristic change observed. A uveitis is less specific but was even more regularly present as indicated by the occurrence of hyperemia and deep radial folds in the iris and a corona of dilated vessels visible around the cornea and extending back over the bulbar con-

TABLE II.
Tests for Eastern Equine Encephalomyelitis in Rabbit Eyes After Injection of 0.2 ml of 10% Infected Mouse Brain.

Rabbit No.	Eye	Material tested	Interval, days	Fate of mice inj. with dilutions			Cornea
				10^{-0}	10^{-1}	10^{-2}	
K510	Rt.	Aq. h.	2	2 2 3	3 4 s	s s s	Opaque
	”	Tissue	”	—	2 2 6	s s s	”
	Left	”	”	2 s s	s s s	s s s	”
K511	Rt.	Aq. h.	4	s s s	s s s	s s s	”
	”	Tissue	”	—	4 s s	s s s	”
	L.	Aq. h.	”	s s s	s s s	s s s	”
K531*	Rt.	”	11	s s s	—	s s s	”
	L.	”	”	s s s	—	s s s	Opaque at 3 days. Cleared

* Severe encephalitis.

junction to a variable extent. Histologically, this uveitis appeared as hyperemia of the iris and ciliary body, usually with very little cellular exudate. The choroid and retina were not visibly affected after injection of either the eastern or western viruses into the anterior chamber.

Conclusion. The corneal edema and opacity resulting from intra-ocular injection of the virus of western equine encephalomyelitis in domestic rabbits occurs only in response to large amounts of virus. It appears to be analogous to the ocular response to influenza virus, which has been attributed to toxic properties of the virus. An entirely similar ocular reaction has been induced by injection of equine encephalomyelitis virus of the eastern type. Limited growth of equine encephalomyelitis virus may occur in the eye but is not a significant factor in the described reaction.

15242

A Vaccine Against Japanese B Encephalitis Prepared from Infected Chick Embryos.

JOEL WARREN AND RALPH G. HOUGH.
(Introduced by J. E. Smadel.)
From the Division of Virus and Rickettsial Diseases, Army Medical School, Army Medical Center, Washington, D.C.

Most of the pathogenic viruses affecting man have been cultivated in the developing embryonated egg.[1] In addition the egg has provided a source of vaccine for immunizing human beings against such viruses as yellow fever,[2] influenza,[3] vaccinia,[4] and equine encephalomyelitis.[5] Although the virus of Japanese B encephalitis was shown to multiply in the embryonated egg,[6] growth of this agent in chick embryo tissue was not sufficiently luxuriant to offer promise of providing material for a potent vaccine against this disease. Indeed, the reported experiments regarding such a vaccine indicated that it possessed little immunizing capacity.[7]

The vaccines at present employed against Japanese B encephalitis are prepared from infected brain tissue of mice.[7] These are capable of eliciting neutralizing antibodies against the virus in vaccinated human beings and of inducing resistance to infection in experimental animals. Such vaccines have been prepared commercially and have been authorized for use in the U. S. Army. Although the mouse brain vaccine has desirable immunological properties, it possesses certain potentially objectionable features. For example, it is well known that vaccines containing brain tissue may on occasion produce a demyelinizing encephalopathy. In addition the technical difficulties inherent in the large scale manufacture of such a vaccine are considerable. Because of these disadvantages it seemed worth while to reinvestigate the possibilities of obtaining a potent vaccine from tissues of infected chick embryos.

---

[1] Burnet, F. M., Med. Research Council, Special Report No. 220, 1936, London.

[2] Theiler, M., and Smith, H. H., *J. Exp. Med.*, 1937, **65**, 787.

[3] Burnet, F. M., and Lush, D., *Brit. J. Exp. Path.*, 1938, **19**, 17.

[4] Goodpasture, E. W., Buddingh, G. J., Richardson, L., and Anderson, K., *Am. J. Hyg.*, 1935, **21**, 319.

[5] Beard, J. W., Finklestein, H., Sealy, W. C., and Wyckoff, R. W., *Science*, 1938, **87**, 490.

[6] Haagen, E., and Crodel, B., *Zent. Bakt.*, Abt. 1, orig., 1938, **142**, 269.

[7] Sabin, A. B., Duffy, C., Warren, J., Ward, R., Peck, J. L., and Ruchman, I., *J. A. M. A.*, 1943, **122**, 477.

The present report describes the development and preparation of such a vaccine which, in its capacity to immunize mice, compares favorably with the commercial Japanese B vaccines currently made from infected mouse tissue.

Cultivation of the Virus. The "Nakayama" strain of virus was established and carried in embryonated eggs by the inoculation of 0.1 cc amounts of infected allantoic fluid or embryo suspension into the chorio-allantoic sac. Death of the embryo occurred in practically all the inoculated eggs between the fourth and the sixth day.

Several experiments were performed to determine the optimal conditions for growth of Japanese B encephalitis virus in the developing egg. The results may be summarized briefly as follows: The amount of virus in the inoculum and the route by which it was injected had no marked effect upon the yield of virus, provided the agent was inoculated beyond the chorio-allantoic membranes. The agent was present in all tissues and fluids of the inoculated embryo, but the embryo regularly yielded more virus than the fluids or membranes. The infectious titers obtained in one experiment illustrate this point; the results were, embryo $10^{-7.0}$, yolk sac $10^{-5.0}$, allantoic fluid $10^{-4.6}$, chorio-allantoic membranes $10^{-4.5}$, and yolk fluid $10^{-3.6}$. The maximum concentration of virus in the embryo or in the allantoic fluid of inoculated eggs was obtained after incubation at 35° C for 48 to 72 hours. It is of interest to note that during the course of serial passage of the virus in eggs, the infectivity of inoculated embryo increased. By the thirtieth passage, the infective titers of embryo suspensions ranged between $10^{-7.5}$ and $10^{-8.5}$, with an average value of about $10^{-7.8}$.

Preparation and Assay of Vaccines. The seed virus to be used for inoculation of eggs was harvested and stored as a 10% suspension of infected embryo tissue in whole filtered chicken serum. Such seed suspensions withstood storage at -70° C in sealed ampoules for more than 1 month with no appreciable loss in titer. Both seed virus and material for vaccine were obtained from embryonated eggs which were 7 or 8 days old when infected via the chorio-allantoic sac. These were subsequently incubated at 35.0° C for 3 days and at the end of this time the embryos were harvested.

The present method for preparing formalinized vaccine from the infected embryo is now given in detail. The whole embryo was removed from groups of 20 to 200 infected eggs. The lens and retinal cup were dissected out and discarded. The remaining embryonic tissue was then pooled and homogenized in a Waring blendor for 5 minutes with sufficient physiological saline, buffered at pH 7.4, to make a 10 or 20% suspension by weight. The resulting suspension was strained through gauze. After samples were removed for sterility tests and for titration of infectivity, sufficient formaldehyde U. S. P. was added to give a final concentration of 0.2%.*

The material was then stored at 5° C for 2 weeks. It was then assayed for potency and a safety test was performed. For the latter test, 10 mice were inoculated intracerebrally with the vaccine and observed for signs of neurological disease for a period of 2 weeks in order to determine that inactivation of the virus had been complete. The protein nitrogen content of seven samples of 20% embryo vaccines was determined; the values ranged between 0.68 mg/cc and 0.95 mg/cc with an average of 0.84 mg/cc. (The protein nitrogen in a 10% suspension

---

* Experiments were performed to determine the comparative effectiveness of formaldehyde and ultra-violet light as inactivating agents on allantoic fluid vaccines. The minimal lethal concentration or exposure time was first determined for each agent. The ultra-violet source was a water-cooled mercury vapor lamp of the type described by Oppenheimer and Levinson.[8] Under the conditions of these experiments formaldehyde-treated vaccine yielded superior antigenic potency. The irradiation experiments were performed in collaboration with Dr. Fred Stimpert of the Parke, Davis Co., Detroit, Mich.

[8] Oppenheimer, F., and Levinson, S. O., cited in *J. Immunol.*, 1945, **50**, 317 (original paper unpublished).

TABLE I.
Japanese Encephalitis.
The Immunizing Capacity of Vaccines Prepared from the Chick Embryo.

Egg passage No.	Type of vaccine	Incubation temp., °C	Infectivity titer before inactivation	Minimal immunogenic dose; 50% end point cc
14	10% embryo	37.5	$10^{-7.0}$	0.57
17	” ”	37.5	$10^{-7.4}$	0.043
22	” ”	35.0	$10^{-7.5}$	0.038
24	” ”	35.0	$10^{-7.3}$	0.060
28	” ”	37.5	$10^{-7.0}$	0.057
31	” ”	37.5	$10^{-7.5}$	0.038
32B-1	20% ”	35.0	$10^{-8.4}$	0.006
32B-2	” ”	37.5	$10^{-7.3}$	0.012
39A-1	” ”	35.0	$10^{-7.5}$	0.009
39A-2	” ”	37.5	$10^{-7.5}$	0.016
39	” ”	35.0	$10^{-8.2}$	0.008
43	” ”	35.0	$10^{-7.7}$	0.0048
43A	” ”	35.0	$10^{-7.8}$	0.0043
Pool 50-52	” ”	35.0	$10^{-7.2}$	0.121
50B	” ”	35.0	$10^{-7.4}$	0.0023
51	” ”	35.0	$10^{-8.0}$	0.0100
51A	” ”	35.0	$10^{-7.3}$	0.008

of infected mouse brain simultaneously tested averaged 1.1 mg/cc.) The method employed in the assays of the Japanese B encephalitis vaccines was a simplified procedure used by A. B. Sabin[9] in routine laboratory tests on experimental vaccines. It differs from the standard method he developed for routine assay of mouse brain vaccines for commercial purposes in that fewer mice are used and the challenge dose of virus is not titrated intraperitoneally. However when assays on chick embryo vaccines were performed at the National Institute of Health using the standard method, the results agreed closely with tests on the same material done at the Army Medical School.

The method we have employed is as follows: Graded amounts of vaccine are inoculated intraperitoneally into groups of 10 mice weighing 18 to 20 g each. The dose is repeated 3 days later. One week after the first dose of vaccine, the mice are injected intraperitoneally with a challenge inoculation consisting of 0.3 cc of a 10% suspension of infected mouse brain. In order for a test to be considered valid the challenge inoculum must have an intracerebral titer of $10^{-8.0}$ or more and must kill at least 80% of 20 non-vaccinated mice which are included in each test as a control. Test mice are observed for a period of 3 weeks and the deaths are recorded. The 50% end-point method of calculation is applied to the data to determine the amount of vaccine required to protect half of the mice against the challenge inoculation. This quantity is designated as the Minimal Immunogenic Dose.

The present standard of potency for Japanese B encephalitis vaccines which has been adopted by the National Institute of Health and by the Office of the Surgeon General, U. S. Army,[10] requires that a total inoculum of not more than 0.01 cc of vaccine protect at least 50% of the vaccinated mice.

Results. Vaccines against Japanese B encephalitis have been prepared from chick embryo which meet the standards acceptable for mouse brain vaccine, see Table I.

It will be noted that the vaccines which meet these requirements all contained 20% infected embryo tissue. Thus, 8 vaccines of a group of 11 which contained 20% tissue assayed 0.01 cc or less, while all 6 of the 10% vaccines were unacceptable. The 10% vaccines were prepared from eggs infected with early passage material, and the infectious

---

[9] Sabin, A. B., personal communication.

[10] Federal Security Agency, National Institute of Health, *Minimum Requirements: Japanese B Encephalitis Vaccine*, Revision of 5 February, 1945.

TABLE II.
Effect of an Adjuvant ("Falba" and Mineral Oil) on the Immunizing Capacity of Certain Japanese B **Vaccines.**

Vaccine tested	M.I.D. vaccine assayed in Saline, cc	M.I.D. vaccine assayed in 2% "Falba"-Min. oil cc
No. 22—10% chick embryo	0.038	0.017
No. 32B—20% chick embryo	0.006	0.0025
Pool II—20% chick embryo—crude	0.011	0.0029
Pool II—20% chick embryo—angle centrifuged	0.013	0.004
No. 43—10% mouse brain	0.040	<0.0012

titers of the tissues used in their manufacture were generally somewhat lower than the titers of tissues which were used for the 20% preparations. That there was some correlation between the infectivity of the embryo tissue and the potency of the vaccine prepared from it, is clearly demonstrated in Table I. Thus, all 5 of the 20% vaccines made from tissues with infective titers greater than $10^{-7.5}$ were acceptable when assayed while only three of the 6 similar preparations with infective titers before inactivation of $10^{-7.3}$ to $10^{-7.5}$ were satisfactory. Further evidence for the border line immunogenic properties of vaccines made from tissues with titers of $10^{-7.0}$ to $10^{-7.5}$ was found in the unsatisfactory results obtained with the 10% vaccines. Finally, several vaccines prepared from chorio-allantoic fluids with titers of $10^{-6.5}$ or less were unacceptable when assayed.

By means of adsorption, centrifugation, and the use of adjuvants, attempts were made to increase the immunizing capacity of the embryo vaccine. Concentration of the antigenic material of the vaccines could not be obtained with adsorption on calcium phosphate or potassium alum. High speed centrifugation in the Sharples† or the angle ultracentrifuge removed most of the antigen from the supernatant fluid of both allantoic fluid and 10% embryo vaccines but the sediment did not exhibit the expected increase in potency.

For example, the sediment obtained on high speed centrifugation of a 10% embryo vaccine was resuspended in sufficient saline solution to make 1/10th the original volume; this had a two- to three-fold increase in immunizing capacity over the original vaccine and contained about one and a half times the amount of protein nitrogen of that in the starting material. Thus, the immunizing agent had been concentrated in the ultra-sediment and perhaps somewhat purified.

A mixture of "Falba"‡ and mineral oil has been used as an adjuvant to augment the immunizing capacity of certain bacterial and protozoal antigens[11] and of influenza vaccines for human beings.[12] We have obtained encouraging results with this adjuvant employed in conjunction with Japanese B embryo vaccines, see Table II.

A mixture of equal parts of "Falba" and mineral oil was prepared and a 2% emulsion of this material in physiological saline solution was used as a diluent for the vaccines throughout the assay procedure. Control assays were done on the same lots in the ordinary manner, *i.e.* dilutions were made in saline solution. It is evident from the results presented in Table II that the minimal immunogenic dose of a given vaccine was appreciably less when the adjuvant was employed. It may be pointed out that vaccines containing "Falba" and mineral oil elicit certain untoward reactions when injected into human beings.[12] For this reason studies are now in progress to determine whether other agents will serve as satisfactory adjuvants with Japanese encephalitis vaccine.

---

† The Sharples centrifugation experiments were performed with the kind assistance of Dr. W. M. Stanley, Rockefeller Institute for Medical Research, Princeton, N.J.

‡ "Falba" is the proprietary name for a compound prepared from lanolin and composed of a mixture of "oxycholestrins and cholestrins."

[11] Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 548.

[12] Henle, W., and Henle, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 179.

At the present time no information is available regarding the capacity of this vaccine either alone or with adjuvants to immunize man.

Summary. 1. Highly infectious suspensions of embryo tissue have been prepared from embryonated chick eggs inoculated with the Japanese B virus. Infective titers of $10^{-7.5}$ to $10^{-8.5}$ were regularly encountered under suitable conditions.

2. Formalinized vaccines made from 20% suspensions of infected chick embryo tissue are capable of immunizing mice against intraperitoneal infection with the virus of Japanese B encephalitis. Such vaccines are comparable in potency to the Japanese B encephalitis vaccines prepared from infected mouse brain and used by the U. S. Army.

15243 P

Cytotoxic Property of Mouse Cancer Antiserum.*

ROBERT G. GREEN.

From the Department of Bacteriology and Immunology, University of Minnesota Medical School, Minneapolis.

In our investigations of the milk factor,[1] or the Bittner virus, associated with mammary carcinoma of mice, an antiserum prepared from rabbits has been tested for its toxicity for cancer cells. In his studies, Andervont[2] found that an antiserum prepared in rabbits by injecting as antigens the centrifugates of mouse tumor, neutralized the milk factor, while normal serums failed to do so. Green, Moosey and Bittner[3] likewise have shown that antiserums prepared from immunized rabbits, as well as from immunized rats, neutralized the agent. We have prepared additional control serums by immunizing rats and rabbits against normal mouse tissue centrifugates, and these have failed to neutralize the virus. This finding demonstrates that the milk factor is antigenically unlike mouse tissue and might more properly be considered a foreign virus. In the experiments here reported a rabbit antiserum prepared against the Bittner virus has been tested for its toxic properties for mammary cancer cells when mixed with them *in vitro*.

Three rabbits were immunized by 5 weekly injections of cancer centrifugate given intraperitoneally. A suspension of mouse cancer tissue was homogenized and subjected to fractional centrifugation. The first sediment, obtained by centrifugation for 1½ hours at 14,000 X gravity, was discarded. The supernatant suspension was again centrifuged 1 hour at 100,000 X gravity. This second sediment was collected, suspended in a 0.9% saline solution and injected in a dosage corresponding to 2 g of original tumor tissue. The rabbits were bled for serum 14 days after the fifth injection of centrifugate. An antiserum against normal lactating mouse mammary tissue was prepared in an identical manner by subjecting lactating breast tissue to the same processes. The normal breast tissue was obtained from mice susceptible to, but not carrying, the Bittner virus. An additional control serum was made from normal rabbit blood. Each of the 3 serums, as finally prepared, represented a pooled serum from 3 rabbits. The 3 serums were test-

---

* Aided by grants from the Cancer Research Fund, Graduate School of the University of Minnesota, and the Jane Coffin Childs Memorial Fund for Medical Research.

[1] Bittner, J. J., *Science*, 1936, **84**, 162.

[2] Andervont, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1944, **5**, 143.

[3] Green, R. G., Moosey, M. M., and Bittner, J. J., *Cancer Research*, 1945, **5**, 588.

TABLE I.
Cytotoxic Property of Mouse Cancer Antiserum.
Rabbit antiserum—C3H tumor—ZBC mice without milk factor.

		No. tumors in			
No. mice	Tumor cell suspension injected with	10	15	40	60 days
10	Cancer antiserum	0	0	0	0
8*	Normal mammary antiserum	1	3	8	8
10	Normal rabbit serum	1	1	5	7
10	Saline sol.	10	10	10	10

* Two died from intercurrent infection.

ed for their comparative cytotoxicity.

To test the serums, a cancer-cell suspension for the production of transplant tumors was obtained as follows. A mass of cancer tissue was forced through a tissue press. The macerated cancer tissue was then suspended in saline solution and allowed to settle for 30 minutes. A heavy sediment of tissue masses settled out and the supernatant suspension of cancer cells and small cellular masses was removed for injection into susceptible mice. The concentrated cancer-cell suspension was then diluted 10 times. To 4 portions of the cell suspension were added, respectively, 5 volumes of cancer antiserum, normal tissue antiserum, normal rabbit serum, and 0.9% saline solution. All mixtures were treated in the same manner. They were held at room temperature 3 hours, and then in a refrigerator at 7° C for another 3-hour period. Each preparation was then injected in a dosage of 0.6 cc, intraperitoneally, into a separate group of 10 mice. The dose given each mouse represented 0.1 cc of a 1 to 10 suspension of cancer cells and 0.5 cc serum or 0.9% saline solution.

The formation of tumors at various intervals is shown in Table I.

It is evident from the data that the cancer antiserum completely inhibited growth of cancer cells. Both the normal rabbit serum and the normal mammary tissue antiserum effected a delay in the development of tumors. The normal rabbit serum appears to have inhibited slightly the growth of tumor cells, since only 7 mice of the 10 injected displayed tumors at the end of 60 days.

Tumors developed in all mice injected with tumor cells in saline solution much more rapidly than they developed in mice receiving cancer cells in normal rabbit serum or in normal mammary tissue antiserum. There appears, therefore, to be something in normal rabbit serum which interferes with the rapid growth of cancer cells. This effect of normal serum is not increased, but possibly decreased, by immunization with normal tissue centrifugates.

These results seem to demonstrate that an antiserum produced from mouse cancer-cell centrifugates is capable not only of neutralizing the virus but also of inhibiting the growth of cancer cells produced by the virus.

Conclusions. An immune serum prepared in rabbits against the mouse mammary carcinoma milk factor, or Bittner virus, when mixed with cancer cells for a period of 6 hours completely inhibits their growth so that transplant tumors do not develop. Control tumors developed after similar treatment of tumor cells with serum from normal rabbits and with serum from rabbits immunized with normal lactating mouse breast tissue.

15244

Antigenic Character of the Cancer Milk Agent in Mice.*

Robert G. Green, Marye M. Moosey, and John J. Bittner.

From the Department of Bacteriology and Immunology and the Division of Cancer Biology of the Department of Physiology, University of Minnesota Medical School, Minneapolis.

The influence of milk in the transmission of mammary cancer in mice described by Bittner[1] was determined by him to be filterable.[2,3] The colloidal dimension of the influence was ascertained by means of ultracentrifugation by Visscher, Green, Bittner, Ball and Siedentopf,[4] and these authors suggested that the agent might be classed as a filterable virus. Additional data showing that the agent is filterable have been presented by Andervont and Bryan[5] and by Bittner, Evans and Green.[6] We have pursued studies on the antigenic character of the milk agent to gain further information as to its general characterization and in particular as to its properties as a virus.

Studies on the antigenic nature of the mouse-tumor agent have been pursued by Andervont and Bryan,[5] who have recently reported on the neutralization of the milk influence by immune serums. Two immune serums were prepared by them, each from one rabbit. These serums apparently neutralized the mouse mammary-tumor milk agent, as only 1 of 36 mice injected with tumor material mixed with the antiserums developed a tumor, while 10 of 15 mice injected with tumor material mixed with normal rabbit serum developed tumors.

In our investigations of the antigenic character of the milk agent, we have utilized as controls the agent suspended in saline solution and the agent treated with normal serum and with serum from animals immunized with normal tissue. As the agent we have used high-speed centrifugates collected between a first run at 15,000 g and a second at 95,000 g. These centrifugates, which were previously demonstrated[4] to contain much of the milk agent, have been injected into rabbits and white rats to produce antiserums. Five doses of centrifugate, each consisting of a 4-g equivalent of spontaneous mouse tumor suspended in 3.5 cc of saline solution, were injected into rabbits at 5-day intervals. The rats were immunized similarly, except that each immunizing dose was 2 cc and contained the equivalent of 1 g of mammary-tumor tissue. The animals were bled for the production of antiserum from 7 to 10 days after the last injection. In the first experiment on the neutralization of the agent, 90 mice were injected in 3 groups of 30 mice each. Group 1 was injected with a centrifugate equivalent to 0.25 g of tumor tissue suspended in 0.5 cc saline solution. At the end of 11½ months, 25 of the 30 mice had developed mammary cancer. The 30 mice in Group 2 were injected with a centrifugate equivalent to 0.25 g of tumor tissue suspended in 0.5 cc of rabbit immune serum after the mixture had stood at room temperature 2 hours. The antiserum was a pooled mixture of the serums of 3 rabbits immunized as described above. At 11½ months, 2 of the 30 mice had developed tumors. The 30 mice in Group 3 were injected with similar amounts of centrifugate suspended in 0.5 cc of normal rabbit serum. Of these, 14 had developed breast cancer at 11½ months. All inoculated mice in these groups were of the

---

* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the Cancer Research Fund of the Graduate School of the University of Minnesota, and the Citizens Aid Society of Minneapolis.

[1] Bittner, J. J., *Science*, 1936, **84**, 162.

[2] Bittner, J. J., *Science*, 1942, **95**, 462.

[3] Bittner, J. J., *Cancer Research*, 1942, **2**, 710.

[4] Visscher, M. B., Green, R. G., Bittner, J. J., Ball, Z. B., and Siedentopf, H. A., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 94.

[5] Andervont, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1944, **5**, 143.

[6] Bittner, J. J., Evans, C. A., and Green, R. G., *Science*, 1945, **101**, 95.

TABLE I.
Neutralization of Mouse Milk Agent with Rat Immune Serum.

No. mice	Tumor centrifugate mixed with	Tumors produced in mo.					
		7½	8	9	10	11½	13½
24	Spontaneous tumor antiserum	0	0	0	0	0	0
24	Transplant tumor antiserum	0	0	0	0	0	0
24	Normal tissue antiserum	1	3	7	10	12	21
24	Normal rat serum	2	4	8	9	12	13
24	0.9% saline sol.	6	10	15	15	17	18

Dosage: Centrifugate equivalent of 0.2 g tumor tissue mixed with 0.18 cc antiserum in 0.9% saline to make total volume of 0.23 cc. The mixtures were held at room temperature for 2 hours before injection.

Note: All injected mice subjected to forced breeding. Mice 1-mo-old ZBC stock without milk influence.

ZBC strain, mice which were susceptible to, but did not carry, the tumor agent. After inoculation, the mice were maintained as forced breeders in order to stimulate maximum cancer development. These results confirm the findings of Andervont and Bryan.[5]

A second experiment on the antigenic properties of the milk agent has yielded similar results 14 months after its inception. The antiserum used to neutralize the milk agent in the latter experiment was prepared from laboratory rats as described above. Three antiserums were prepared: one from spontaneous tumor tissue; one from transplanted tumor tissue; and one from normal mouse tissue consisting of heart, liver, spleen and kidney. Each antiserum, as finally prepared, was a pooled serum from 3 immunized rats. In addition, a pool of normal rat serum was prepared by combining the serums from 3 uninjected rats. ZBC mice not carrying the milk influence were injected at 1 month of age in groups of 24 with centrifugates of tumor tissue mixed and incubated for 2 hours with the various antiserums. One control group received infective centrifugate suspended in 0.9% saline solution. The appearance of tumors at different intervals and the number ultimately produced are shown in Table I.

Of the 2 groups of mice totaling 48 that received spontaneous tumor antiserum and transplanted tumor antiserum, none has developed cancer at 13½ months. Twenty-one mice of the 24 receiving normal tissue antiserum have developed cancer, and 13 of the 24 that received the normal rat serum have developed cancer. Of the 24 control mice, which received the infective centrifugate without the addition of serum, 18 have become cancerous. These additional data obtained with antiserums made from the closely related rat would seem to establish rather definitely that the infective agent in mouse mammary carcinoma is antigenic.

Demonstration that the milk influence of Bittner is an antigen becomes of greatest importance in its characterization. First termed an *influence* and later an *agent* or an *incitor*, its antigenic capacity, together with other attributes, would seem to put it in the class of filterable viruses. Although it is difficult to describe a virus accurately, its most definitive characters are: (1) reproduction only in association with living host cells, (2) small size, usually ultramicroscopic, and (3) antigenic capacity. All 3 basic criteria are now known to be met by the milk agent. Inasmuch as many tumors in animals are known to be virus infections, it would seem reasonable from the evidence at hand to consider the mammary cancer of mice an infectious process and its cause a filterable virus.

The antiserum produced in rats by the injection of normal mouse tissues failed to produce any adverse effect upon cancer-formation by the agent. Of the 3 groups of controls, those receiving the normal tissue antiserum have presented the largest number of cancerous mice. This indicates that the agent is antigenically different from mouse tissue and is not derived therefrom.

Normal rabbit serum and normal rat serum both seem to contain something that is antagonistic to the cancer agent. Both normal serums effected a retarding of tumor development to a rate below that observed with the agent in saline solution, and ultimately a smaller number of tumors was produced.

Conclusions. The milk factor of mammary mouse carcinoma is highly antigenic and stimulates the formation of antibodies in both rabbits and rats. The mouse cancer antiserum neutralizes and renders inactive mouse cancer centrifugates. Equally effective antiserums are prepared from spontaneous and from transplant tumors. Normal rabbit and rat serums have a slight effect in inactivating the mouse cancer virus. The finding that antiserum against normal mouse tissue does not have any neutralizing or adverse effect on the milk agent indicates that the agent is a virus of exogenous origin.

15245

Cell-Blockade in Canine Distemper.*

Robert G. Green and Cyril S. Stulberg.

From the Department of Bacteriology and Immunology, University of Minnesota Medical School, Minneapolis.

The interference phenomenon of animal viruses was first observed by Hoskins[1] and by Magrassi[2] independently. Magrassi observed an interference between nonencephalitic and encephalitic herpes viruses in rabbits, and Hoskins reported an interference between neurotropic and pantropic yellow fever viruses in monkeys. Subsequent investigations by Findlay and MacCallum,[3] Dalldorf,[4] Jungeblut and Sanders,[5] Ziegler, Lavin and Horsfall,[6] the Henles,[7] and others have shown that the cell-blockade, or interference phenomenon, can occur in many animal viruses under experimental conditions.

We have conducted experimental studies of simultaneous infections of young foxes with virulent distemper virus and a distemper virus modified by ferret passage, and found that the phenomenon of interference, or cell-blockade, can be demonstrated for these viruses. Foxes inoculated *intranasally* with virulent distemper virus can be protected from fatal infection by subsequent inoculation of the ferret-passage distemper virus, which is of low virulence for foxes and dogs. In foxes injected *intramuscularly* with the virulent distemper virus, the course of the infection is also blocked by the introduction of the modified virus either 3 days before, or at the same time that, the virulent distemper virus is given. However, if the interval elapsing between the intramuscular injection of the virulent virus and the subsequent inoculation of distemperoid virus is as long as 3 days, there is very little interference with the course of the virulent infection.

Materials and Methods. The animals inoculated were red fox pups 10 to 16 weeks old. In any one experiment the foxes were very nearly the same age. The virulent distemper virus was of dog origin, and had been passed experimentally through foxes until it acquired an extremely high virulence for that animal. The ferret-passage virus was the distemperoid virus described by Green.[8]

---

* Aided by a grant from the Medical Research Fund of the Graduate School of the University of Minnesota and by experimental facilities and financial support furnished by Fromm Laboratories, Inc., Grafton, Wisc.

[1] Hoskins, M., *Am. J. Trop. Med.*, 1935, **15**, 675.

[2] Magrassi, F., *Z. f. Hyg. u. Infektionskr.*, 1935, **117**, 501, 573.

[3] Findlay, G. M., and MacCallum, F. O., *J. Path. and Bact.*, 1937, **44**, 405.

[4] Dalldorf, G., *J. Imm.*, 1939, **37**, 245.

[5] Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1942, **76**, 127.

[6] Ziegler, J. E., Jr., Lavin, G. I., and Horsfall, F. L., *J. Exp. Med.*, 1944, **79**, 379.

[7] Henle, W., and Henle, G., *Am. J. Med. Sci.*, 1944, **207**, 705.

[8] Green, R. G., *Am. J. Hyg.*, 1945, **41**, 7.

TABLE I.

Interference in Foxes between Virulent Distemper Virus Inoculated intranasally and Distemperoid Virus Inoculated Intramuscularly.

(Exp. 1.)

Days after injection of distemper virus	Group 1 a 10 foxes inj. with virulent distemper virus (Controls)		Group 2-a 10 foxes inj. simultaneously with virulent distemper virus and distemperoid virus‡	Group 3-a 10 foxes inj. with distemperoid virus 3 days after virulent distemper virus‡	Group 4-a 9 foxes† inj. with distemperoid virus 12 days after virulent distemper virus‡
	Sick	Deaths	Sick	Sick	Sick
16	1/10*		2/10		
17	1/10		2/10		
18	1/10		2/10		
19	2/10		2/10		1/9
20	3/10		2/10		1/9
21	4/10		3/10		2/9
22	4/10		3/10		2/9
23	6/10		5/10	2/10	4/9
24	6/10		5/10	2/10	4/9
25	6/9	1	5/10	2/10	4/9
26	6/9		5/10	2/10	4/9
27	6/9		2/10	2/10	3/9
28	6/8	1	2/10	2/10	3/9
29	7/7	1	2/10	2/10	3/9
30	6/6	1	2/10	2/10	3/9
31	2/2	4	0/10	0/10	1/9
32	2/2				0/9
33	2/2				
34	2/2				
35	1/1	1			
36		1			

* The denominator indicates the number of foxes living, and the numerator the number sick on any given day.

† One fox in this group had a broken leg and was killed.

‡ No deaths.

The virulent virus was injected in a dosage of 100 mg and the distemperoid virus in a dosage of 200 mg. All animals were observed daily for symptoms of distemper. When deaths occurred, the disease was identified by the presence of cytoplasmic inclusions in the epithelial cells of the bladder or the trachea.

Interference following intranasal inoculation with virulent virus. Forty red fox pups were divided into groups of 10. Group 1-a, which received virulent distemper virus only, was inoculated intranasally. Animals in this group exhibited symptoms of distemper by the 16th day, and all died of distemper between the 25th and 36th days. The other 3 groups received the same virus intranasally but were given distemperoid virus in addition. Group 2-a received simultaneous inoculations of the two viruses. Two foxes showed symptoms on the 16th day, and by the 23rd day 5 showed mild signs of the disease. All animals had recovered by the 31st day. Group 3-a was given the distemperoid virus 3 days after injection of the virulent virus. Only 2 animals in this group displayed symptoms, which were discernible on the 23rd day. Both were normal by the 31st day. Group 4-a was given distemperoid virus 12 days after receiving the virulent virus. Mild symptoms of distemper were shown by one animal in this group on the 19th day and by 4 animals on the 23rd day. All had recovered by the 32nd day. Details regarding the occurrence of symptoms, deaths, and recoveries are shown in Table I.

These results demonstrate a very decisive interference, or blocking effect, of distemperoid virus on the course of a virulent distemper infection. They show also that a virulent infection corresponding to the natural

TABLE II.
Results of Intramuscular Inoculations of Foxes with Virulent Distemper Virus and Distemperoid Virus.
(Exp. 2).

Days after inj. of virulent distemper virus	Group 1-b 10 foxes inj. with virulent distemper virus (Controls) Sick	Deaths	Group 2-b 10 foxes inj. with distemperoid virus 3 days before virulent distemper virus	Group 3-b 10 foxes inj. simultaneously with virulent distemper virus and distemperoid virus Sick	Deaths	Group 4-b 10 foxes inj. with distemperoid virus 3 days after virulent distemper virus Sick	Deaths
12			Normal				
13	1/10		''			1/10	
14	3/9	1	''			all	1
15	5/9		''			''	2
16	5/9		''			''	3
17	all	1	''			''	2
18	''	1	''	2/10			2
19	''		''	2/10			
20	''	1	''	2/10			
21	''	2	''	1/10	1		
22	''	1	''	0			
23	''	1	''	0			
24	''	1	''	0			
25			''	0			
26			''	0			
27			''	0			
28			''	0			
29			''	0			
30		1	''	0			

disease can be blocked off if distemperoid virus is injected during the incubation period or in the early stage of the generalized infection.

Interference following intramuscular injection of virulent virus. In our second experiment 40 young foxes, in groups of 10, were injected intramuscularly with virulent distemper virus. All 4 groups received the virulent distemper virus at the same time, and 3 of the groups were subsequently given intramuscular injections of distemperoid virus at varying intervals.

Group 1-b was injected with virulent distemper virus only. On the 13th day after the inoculations, the animals began to show symptoms. By the 17th day all the animals were sick, and at the end of 30 days all had died of typical distemper. Group 2-b received distemperoid virus 3 days before the inoculation of virulent distemper virus. These animals remained well, showing no symptoms whatsoever of distemper. Group 3-b was inoculated with virulent distemper virus and distemperoid virus simultaneously. Two animals exhibited symptoms on the 18th day after inoculation. One of them died on the 21st day, while the other recovered. The rest of the foxes in this group showed no symptoms of distemper. Group 4-b received distemperoid virus 3 days after the virulent distemper virus. All the animals showed symptoms by the 14th day, and all died of typical distemper by the 18th day. Details of the occurrence of symptoms, deaths, and recoveries are shown in Table II.

In the third experiment 40 young foxes were also used in groups of 10. The control group received only virulent distemper virus. The other 3 groups were inoculated intramuscularly, first with virulent distemper virus and then with distemperoid virus, which was given to the respective groups 3, 8, and 12 days later.

Group 1-c, the controls, began to show symptoms on the 14th day after the injection of virulent virus. All the animals died of distemper by the 29th day. Group 2-c, which received the distemperoid virus 3 days after the virulent virus, began to exhibit symptoms of distemper on the 12th day. By the 36th day 8 animals had died and 2 had re-

TABLE III.
Results of Intramuscular Inoculations of Foxes with Virulent Distemper Virus and Distemperoid Virus.
(Exp. 3.)

Days after inj. of distemper virus	Group 1-c 10 foxes inj. with virulent distemper virus (Controls)		Group 2-c 10 foxes inj. with distemperoid virus 3 days after virulent distemper virus		Group 3-c 10 foxes inj. with distemperoid virus 8 days after virulent distemper virus		Group 4-c 9 foxes* inj. with distemperoid virus 12 days after virulent distemper virus	
	Sick	Deaths	Sick	Deaths	Sick	Deaths	Sick	Deaths
12			4/10				2/9	
13			5/10				2/8	1
14	4/10		8/10		4/10		3/7	1
15	6/10		9/10		5/10		3/7	
16	all	2	all	3	5/10		3/7	
17	"		"		6/10		all	2
18	"		"		6/10		"	1
19	"	1	"		5/9	1	"	2
20	"	3	"	2	5/9		"	
21	"	1	"	1	4/7	2	"	
23	"		"		4/6	1	"	1
24	"		"		4/6			1
25	"	1	"		all	1		
26	"	1	"		"			
27	"		"		"	1		
28	"		"		"	1		
29		1	"		"			
30			"		"	1		
31			"	1	"	1		
36			0/2	1	0/1			
Total No. deaths		10		8		9		9

* One fox in this group had a broken leg and was killed.

covered. Group 3-c received the distemperoid virus 8 days after the virulent virus. Symptoms first appeared on the 14th day. Nine animals were dead by the 31st day, and 1 had recovered. Group 4-c, inoculated with the distemperoid virus 12 days after the inoculation of virulent virus, began to show symptoms on the 12th day. All animals in this group were dead by the 24th day. Details of the symptoms, deaths, and recoveries are shown in Table III.

The first experiment on the intramuscular injection of virulent virus demonstrated that the distemperoid virus completely protected foxes when it was inoculated before the virulent distemper virus and gave almost complete protection when the inoculations were simultaneous. Both of the experiments on intramuscular injections included a group inoculated with distemperoid virus 3 days after the virulent virus was given. In both cases the controls died within approximately the same length of time. Some differences were observed in the 3-day groups. In Experiment 2 all the animals died but in Experiment 3 only 8 of 10 animals died. The recovery of 2 animals appeared due to protection, since foxes seldom recover once they have exhibited symptoms of virulent distemper. In the second intramuscular experiment little or no protection was apparent in the 8-day group and no protection in the 12-day group. A comparison of the results obtained from intranasal and intramuscular inoculations of virulent distemper virus is shown in Table IV.

Discussion. Our results seem to show that when the virulent distemper virus is inoculated *intranasally*, an injection of the modified virus given at the same time or 3 to 12 days later, so interferes with, or blocks off, the virulent infection that severe symptoms do not develop and recovery is rapid and complete.

TABLE IV.
Comparison of Intramuscular and Intranasal Inoculations of Foxes with Virulent Distemper Virus in Interference Experiments.[*]

Inocula	40 foxes inj. intramuscularly			40 foxes inj. intramusc.			40 foxes inj. intranasally		
	No. injected	No. sick	No. dead	No. injected	No. sick	No. dead	No. injected	No. sick	No. dead
Virulent distemper virus only	10	10	10	10	10	10	10	10	10
Distemperoid virus 3 days before distemper virus	10	0	0						
Distemperoid virus and distemper virus simultaneously	10	2	1				10	5	0
Distemperoid virus 3 days after distemper virus	10	10	10	10	10	8	10	2	0
Distemperoid virus 8 days after distemper virus				10	10	9			
Distemperoid virus 12 days after distemper virus				9[†]	9	9	9[†]	4	0

[*] All distemperoid virus injected intramuscularly.

[†] One fox had a broken leg and was killed.

The results obtained in the case of *intramuscular* inoculation of the virulent virus are similar, provided the modified virus is given before, or even at the same time as, the virulent virus. A striking difference, however, is observed in the number of recoveries of treated foxes when the modified virus is given after intramuscular injection of virulent virus. In this case, little or no interference follows the injection of the modified virus.

It appears that protection occurred when the modified virus was given an opportunity to reach tissue cells ahead of the virulent virus. On the other hand, if the virulent virus was able to invade a majority of susceptible cells before the modified virus, no protection occurred. In the intramuscular inoculations the virulent virus, when given first, seems to have immediately entered the blood stream as free virus and to have been distributed rapidly to large numbers of tissue cells. However, in the intranasal inoculations, the virulent virus appears to have invaded the animal body slowly, perhaps principally through neighboring tissue cells. At least, it did not seem to have become free virus in the blood stream and to have invaded large numbers of tissue cells ahead of the later-injected modified virus. The mechanism appears to be a reciprocal cell-block. Either the modified virus will be blocked by the virulent virus, if the virulent virus has already invaded the susceptible cells; or the modified virus, if it gets to the tissue cells first, will block the effect of the virulent virus on those cells. All our experimental results point to a mechanism of cell-blockade rather than to interference by one virus directly with the other.

Summary. A modified ferret-passage distemper virus exhibits an interference, or cell-blockade, phenomenon with respect to a virulent distemper virus in foxes. If the virulent virus is inoculated intranasally, as occurs in a natural infection, interference occurs when the modified virus is inoculated at the same time as, or after, the virulent virus. If the virulent virus is inoculated intramuscularly, the virulent infection can be blocked off by the modified virus only if the modified virus is given before, or at the same time as, the virulent virus. After an intramuscular injection of a virulent virus, any effect of the modified virus is in turn blocked off. The results appear to be determined by the virus that seeds the most tissue cells first. In the case of intranasal inoculation, the distemperoid virus seems to have a definite therapeutic effect during the incubation period and in the stage of early symptoms.

15246

Hydroxybenzene Compounds as Cytoplasmic Fixatives.*

ROBERT A. HUSEBY.† (Introduced by John J. Bittner.)

From the Division of Cancer Biology, Department of Physiology, University of Minnesota, Minneapolis.

Because of a renewed interest in cytoplasmic components quickened by the development of methods for centrifugal fractionation of these constituents, this investigation was undertaken in an effort to develop a simple yet effective histological method for the study of the cell cytoplasm and, more particularly, of such structures as the mitochondria. At the present time 3 types of agents[1] are employed in fixation of the cytoplasm; heavy metals, osmium tetroxide, and formaldehyde. As the protein precipitating properties of the heavy metals have been investigated rather extensively, further consideration of these compounds did not appear to be warranted. Osmium tetroxide gives adequate preservation of only a small layer of cells within a tissue block, its "fixing" properties possibly being due to its action as a strong oxidant. The uncontrollable nature of such oxidation, however, severely limits the usefulness of this reaction in the fixation of tissues. Formaldehyde, on the other hand, is a weak reducing agent, and although in itself it does not yield exceptional results, suggests that other reducing compounds might be of value in the preservation of cytoplasmic constituents.

In line with this reasoning, trihydroxybenzene or pyrogallol was considered as it seemed to be well suited for our purposes, being a relatively strong reducing substance, when in alkaline solution, as is required for the maintenance of cytoplasmic detail. Preliminary investigations revealed that weak solutions of pyrogallol preserved the mitochondria but caused excessive alteration of other cell structures. As hydroxybenzene compounds combine with formaldehyde, pyrogallol-formol mixtures were then tried and found to give more desirable results. Investigation of the unsubstituted dihydroxybenzenes in combination with formaldehyde revealed that hydroquinone and catechol give rather poor fixation but that resorcinol approximates pyrogallol in effectiveness.

Pyrogallol-formol Mixtures. As alkaline solutions of pyrogallol and formaldehyde take up oxygen readily and acid solutions form high polymers on standing, all solutions must be made up shortly before use. This is a relatively minor disadvantage, however, since weighed amounts of pyrogallol can be stored in the dry vials in which the tissue is to be fixed, and the desired amount of alkalinized formaldehyde solution added just prior to use.

For the fixation of tissue pieces, up to 5mm in thickness, solutions of 3.5 to 7% pyrogallol dissolved in 4% formaldehyde alkalinized with 0.1 cc of N. NaOH per 10 cc give the best results. Fixation is complete in 6 to 8 hours but periods of fixation as long as 24 hours do not appear to be particularly harmful. When small tissue fragments or thin layers of cells, such as those obtained in tissue culture, are to be fixed, the time of fixation must be reduced, 15 to 30 minutes sufficing in the latter case if the plasma clot has been removed. After fixation, the tissues can be immediately dehydrated in varying strengths of alcohol and imbedded in paraffin or paraffin mixtures. If the tissues are allowed to stand in 70 to 80% alcohol for periods of weeks or months, they darken considerably and harden somewhat, and this has been found to interfere, to some extent, with certain of the staining reactions.

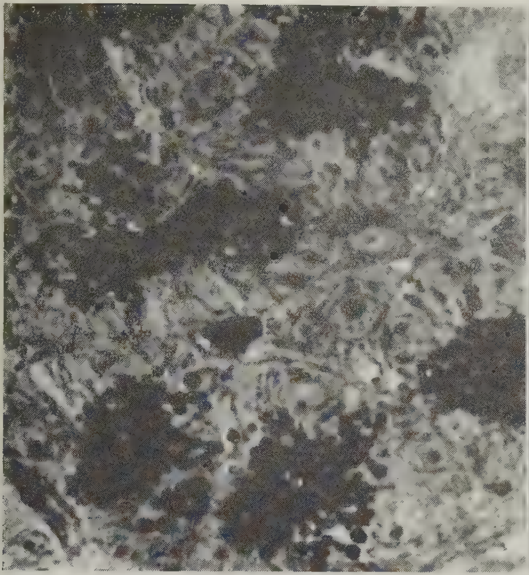
Tissue blocks, prepared as outlined, section easily, and may be cut at thicknesses of

---

* This research was begun in the laboratories of Pathology and Bacteriology of the Rockefeller Institute for Medical Research.

† Fellow of the International Cancer Research Foundation.

[1] Baker, J. R., *Cytological Technique,* London, Methuen, 1933, p. 131.



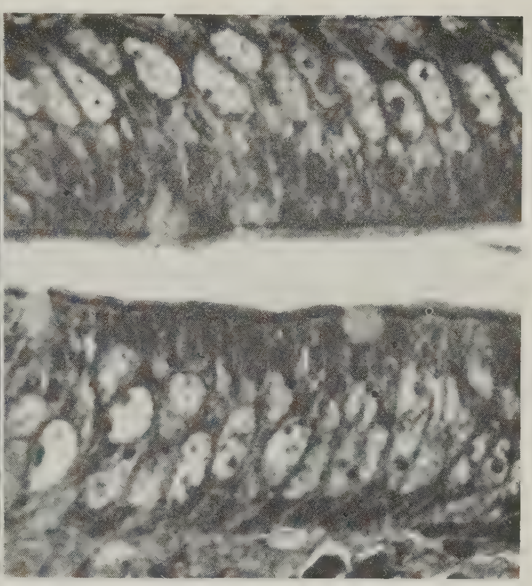


Fig. 1.

A section of pancreas of a non-fasted adult guinea pig showing the centrally located zymogen granules and the more peripherally located elongated mitochondria. Fixed in 9% resorcinol in 4% alkalinized formaldehyde, sectioned at 2 micra, and stained by the Altmann-Bensley procedure. Mag. × 2100.

Fig. 2.

A section of epithelium from the duodenum of a non-fasted mouse showing particularly the mitochondria and the brush border of the cells. Fixed in 7% pyrogallol in 4% alkalinized formaldehyde, sectioned at 2 micra, and stained by the Altmann-Bensley procedure. Mag. × 2100.

Photomicrographs taken by H. W. Morris.

1 to 2 micra without much difficulty. The reaction of various staining procedures following this fixation is interesting. The iron-hematoxylin methods give the best results. Mitochondria and other cytoplasmic granules stain sharply, and the nuclei show good detail as do such structures as basement membranes, terminal bars, and brush borders. The Altmann-Bensley staining procedure also gives good results if the tissue slices are not treated with potassium permanganate and oxalic acid or with potassium dichromate. Schleicher's modification of Mallory-Heidenhain connective tissue stain[2] gives fairly good results although the azocarmine overstains to some extent, but routine hematoxylin and eosin methods must be preceded by treatment with potassium permanganate and oxalic acid to insure staining with the hematoxylin. After the tissues have been stored in alcohol for some time and have darkened considerably, the results obtained with all of the above staining procedures, except iron-hematoxylin, are less satisfactory.

Resorcinol-formol Mixtures. The procedure for the preparation of resorcinol-formol mixtures is the same as that for the pyrogallol-formol solutions already outlined. Tissue pieces are well fixed in solutions of 4.5 to 9% resorcinol in 4% alkalinized formaldehyde. The time necessary for fixation and the subsequent treatment of the tissue blocks are the same as for the pyrogallol-formol mixtures, but tissue fixed with resorcinol-formol darkens much more slowly when stored in alcohol so that storage may be somewhat longer without altering the staining reactions.

Tissue prepared in this manner is somewhat softer and sections with greater ease than when fixed with pyrogallol-formol solutions. Their reaction to various staining procedures is, however, rather different. The Altmann-Bensley method gives the best results for mitochondria. The tissue slices should not be treated with potassium permanganate and oxalic acid, and, although good results are obtained if potassium dichromate is employed, somewhat clearer staining is produced by omitting this step. Routine hematoxylin and eosin methods, Schleicher's modification of Mallory-Heidenhain connective tissue stain, and Pappenheim's methyl green-pyronin method give good results following resorcinol-formol fixation. However, although the nuclei and cytoplasmic ground substance stain well with iron-hematoxylin, the mitochondria do not show up distinctly even after the tissue has been allowed to stand in alcohol until it has darkened.

---

[2] Schleicher, E. M., *Am. J. Clin. Path.* (Tech. Supl.), 1943, **7**, 35.

Discussion. Some investigation of the action of these hydroxybenzene-formol mixtures has been carried out, and the results warrant terse mention here. Although the pH of any fixative is of utmost importance where the cytoplasm is concerned, this aspect is easily adjusted in the solutions outlined above, for, with the addition of NaOH, the weakly acidic pyrogallol and resorcinol form buffer systems. The point of greatest buffering power of these systems is in the neighborhood of pH 9, but with the relatively high concentrations used for fixation, 7% pyrogallol for instance, increasing the concentration of NaOH from .01 N to .02 N only increases the pH from 7.4 to 7.7 and the latter is not excessive for tissue fixation.

The effect of these solutions upon cytoplasmic extracts is also of some interest. Although neutralized formaldehyde does not cause precipitation of the proteins in such extracts and even protects them from subsequent precipitation with alcohol in concentrations of 50% or less, the hydroxybenzene-formol mixtures cause an immediate and heavy precipitation of the protein components. When purified preparations of mitochondria are so treated, the particles agglutinate, but microscopic examination reveals that the individual elements are not appreciably altered morphologically. That they have been chemically modified, however, is evidenced by the fact that after treatment they are insoluble in NaOH even in concentrations of 0.5 N whereas untreated mitochondria dissolve readily in solutions of NaOH that exceed 0.05 N.

The reaction of cells in tissue culture was studied by the method of dark-ground illumination as given by Strangeways and Canti.[3] As the fixing solutions were drawn across the cells, the cell boundaries became sharply demarcated and, although a few of the finer processes were seen to retract, in general the original outlines of the cells were well maintained. The ground substance of the cells became much more brilliantly illuminated, but no discrete particles of precipitated protein nor obvious precipitation patterns appeared. The perinuclear refractile bodies, which probably represent the Golgi apparatus,[4] showed little or no tendency to fuse, and apparently were essentially unaltered as they dissolved completely when 95% alcohol was drawn through the chamber. The mitochondria became less noticeable because of the increased density of the illumination of the ground substance, but careful inspection revealed no evidence of change in their size or shape either with fixation or with subsequent treatment with alcohol and ether. No alteration of size or shape of the nuclei or nucleoli was noted, although the same general increase in density of illumination that was observed in the cytoplasm was also seen in these structures. In general, then, direct observations of tissue culture cells during fixation and subsequent treatment with alcohol and ether substantiated the impression gained from a study of sectioned material that these fixatives distort the cell very little and preserve the mitochondria very well.

The results obtained with these fixing solutions have been especially satisfactory because, in addition to the preservation of cytoplasmic detail, such structures as brush borders, terminal bars, elastic membranes in the arterial system, and basement membranes have been well shown in such preparations. Red cells, however, appear badly distorted in the larger vessels although they are rather well preserved in the capillaries and arterioles. This deleterious effect is probably not of osmotic origin as it occurs when iso-osmotic solutions of hydroxybenzene-formol are used as well as when .85% NaCl is added to such solutions. Another undesirable feature of these fixatives is that when tissue blocks remain in them sufficiently long to allow penetration and fixation throughout the block, the cells near the surface are over fixed. This over fixed zone is, however, not very thick when the tissue has been fixed 6 to 8 hours and does not, as a rule, interfere with the study of the tissue.

---

[3] Strangeways, T. S. P., and Canti, R. G., *Quart. J. Micro. Sci.*, 1927, **71**, 1.

[4] Porter, K. R., Claude, A., and Fullam, E. F., *J. Exp. Med.*, 1945, **81**, 233.

Summary. Methods for the fixation of the cell cytoplasm employing pyrogallol or resorcinol in neutralized formaldehyde solution are outlined. Tissue pieces fixed with these solutions have, in general, shown few signs of distortion, and the protein cytoplasmic elements of the cells have appeared well preserved, such structures as mitochondria, secretion granules, and some of the specific types of granulation being well maintained. The advantages of these methods lie in the simplicity and rapidity of the procedures, the ease with which the tissue can be sectioned at a thickness of 1 to 2 micra, and the generally satisfactory results obtained.

15247

Antipyridoxine Activity of 2, 4-Dimethyl-3-Hydroxy-5-Hydroxymethylpyridine in the Chick.

Walther H. Ott. (Introduced by H. Molitor.)

From the Merck Institute for Therapeutic Research, Rahway, N.J.

In the course of investigations on the vitamin activity of pyridoxine analogs, it was discovered that 2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine had very strong antipyridoxine properties. Growth and survival of pyridoxine-deficient chicks in curative assays were used as the criteria of response. This substance, a desoxypyridoxine, had been studied in pyridoxine-deficient rats by Möller[1] and associates and by Unna,[2] both of whom found no pyridoxine activity. No indication of antipyridoxine activity was reported by these investigators.

Day-old female single comb White Leghorn chicks were fed a commercial starting ration for 3 days and then were given a purified diet (Table I) deficient in pyridoxine. After 6 days on this deficient diet, when many chicks were beginning to lose weight, the chicks were divided into groups of 7 selected in such a way that all groups were as nearly alike as possible in weights of the individual chicks. The test substance was given in single oral doses to each chick on the 11th day of age and on the following 3 alternate days. Three or more dose levels of pyridoxine in the range from 0 to 24 μg per dose were given in each assay to establish the standard curve of response for that assay. New standard solutions of pyridoxine and of desoxypyridoxine were prepared in water for each assay and kept under toluene in a refrigerator for the 6-day dosing period. The chicks were weighed before each dose and on the second day after the last dose was given. The pyridoxine-deficient diet was fed to all groups during the assay period.

Two 100 μg doses of desoxypyridoxine were fatal to pyridoxine-deficient chicks even when each dose was preceded by a 16 μg dose of pyridoxine (Table II). Furthermore, a level of the analog as low as 16 μg per

---

[1] Möller, E. F., Zima, O., Jung, F., and Moll, Th., *Naturwiss.*, 1939, **27**, 228.

[2] Unna, K., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 122.

TABLE I.
Pyridoxine-Deficient Diet for Chicks.

Dextrose (cerelose)	46.5
Casein (vit.-free)	25.0
Gelatin	10.0
Calcium gluconate	5.0
Salts IV[3]	5.0
KH_2PO_4	1.0
Liver extract ‘‘L’’*	2.0
Wheat germ oil	4.5
400D fish liver oil	0.5
Cystine	0.2
Choline	0.2
Inositol	0.1
p-Aminobenzoic acid	0.03
Niacin	0.01
Calcium pantothenate	0.004
Riboflavin	0.002
Thiamine	0.002
Menadione	0.0004
Biotin	0.00004
Total	100.05

* Wilson & Co., Inc., Chicago, Ill.

dose also proved fatal to the birds. On the other hand, pyridoxine-deficient chicks which received no supplement suffered less than 60% mortality in the same assays.

That the action of the analog was probably due to interference with pyridoxine activity rather than to some other type of toxicity was indicated by a test on chicks receiving a commercial starting ration. Dose levels of 50, 100, and 200 μg were given to groups of 3 six-day-old chicks each and repeated on the two following alternate days. All chicks survived, and no retardation of growth was observed. These chicks, with normal body stores of pyridoxine and an adequate supply of the vitamin in the commercial starting ration, were able to tolerate total dosages of at least 600 μg of the analog, a quantity more than 6 times as great as the lethal dose to more than half the pyridoxine-deficient chicks.

On the assumption that **desoxypyridoxine** acted as an antivitamin, further assays were conducted with pyridoxine-deficient chicks on the pyridoxine-deficient diet to estimate the amount of the analog needed to counteract a given amount of pyridoxine (Table III). In these tests the dose of pyridoxine always preceded the dose of the analog by 15 to 30 minutes.

The growth response of each group receiving both compounds was compared with the standard curve of response from graded levels of pyridoxine, and the net pyridoxine activity of each combination was estimated. Subtraction of the observed pyridoxine activity from that theoretically expected on the basis of the level of pyridoxine administered gave the apparent loss in pyridoxine activity occasioned by the dose of desoxypyridoxine. From these last 2 values it can be seen that about 2 parts of the analog counteracted the biological

TABLE II.
Activity of Desoxypyridoxine and Pyridoxine in 9-day Curative Tests with Chicks Receiving a Diet Deficient in Pyridoxine.

Oral supplement*			
Pyridoxine hydrochloride μg	Desoxypyridoxine hydrochloride μg	Survival of chicks, alive/total	Wt gain per chick, g
—	100	0/7	(5 dead after 1st dose) (2 ’’ ’’ 2nd ’’)
—	—	6/7	3.8
16	—	7/7	36.6
16	100	0/7	(4 dead after 1st dose) (3 ’’ ’’ 2nd ’’)
—	—	4/7	0.7
16	—	7/7	33.0
—	16	0/6	(3 dead after 1st dose) (2 ’’ ’’ 2nd ’’) (1 ’’ ’’ 3rd ’’)
—	—	3/7	—7.4
16	—	6/6	32.0

* Amt given per chick on each of 4 successive alternate days.

---

[3] Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

TABLE III.
Antipyridoxine Activity of Desoxypyridoxine as Measured in 9-day Curative Assays with Chicks Receiving a Pyridoxine-Deficient Diet.

Oral supplement*					
Pyridoxine hydrochloride, µg	Desoxypyridoxine hydrochloride µg	Survival of chicks, alive/total	Wt gain per chick, g	Loss in pyridoxine activity, µg	Ratio of inhibition, analog : vit.
16	32	2/7	15.5	11	3:1
16	16	6/7	20.5	7	2:1
16	8	7/7	18.8	8	1:1
4	—	6/7	14.5		
8	—	6/7	19.5		
16	—	7/7	28.0		
16	16	5/7	23.4	3	5:1
24	16	7/7	30.2	8	2:1
35	50	6/7	21.4	23	2:1
—	—	3/7	7.2		
8	—	6/7	16.1		
12	—	7/7	19.6		
16	—	6/6	32.0		

* Amt given per chick on each of 4 successive alternate days.

activity of one part of pyridoxine (Table III).

Previously reported B vitamin antagonists are relatively much weaker antivitamins. For example, only one molecule of thiamine was required to overcome the antithiamine activity of 40 molecules of pyrithiamine in mice,[4] and a similar ratio was found between riboflavin and isoriboflavin rats.[5] It therefore appears that desoxypyridoxine is the most potent of the B vitamin inhibitors as yet discovered.

Summary. Bioassays with chicks have shown that 2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine is a very potent inhibitor of pyridoxine. Under the conditions of these experiments, two molecules of the inhibitor were sufficient to offset the vitamin activity of one molecule of pyridoxine. This ratio was found to hold for suboptimal and optimal amounts of pyridoxine given to pyridoxine-deficient chicks.

---

[4] Woolley, D. W., and White, A. G. C., *J. Biol. Chem.*, 1943, **149**, 285.

[5] Emerson, G. A., and Tishler, M., Proc. Soc. Exp. Biol. and Med., 1944, **55**, 184.

15248

Adrenolytic and Sympatholytic Actions of Priscol (benzyl-imidazoline)

Dorothy Chess and Fredrick F. Yonkman.

From the Department of Pharmacology, Research Division, Ciba Pharmaceutical Products, Inc., Lafayette Park, Summit, N.J.

Experimentally employed adrenolytic agents include ergotoxine, ergotamine tartrate, yohimbine hydrochloride, various esters of yohimbic acid and the Fourneau Compounds, F933 and F899. In some respects these agents seem to be sympatholytic as well as adrenolytic. It has been reported[1] that Priscol exerts this dual effect.

The adrenolytic properties of priscol have been corroborated[2] in respect to alterations in blood pressure in urethanized cats and it seemed desirable to test the drug's sympatholytic properties. This was done not only in relation to general circulatory responses but also in reference to other functions under control of the sympathetic nervous system.

---

[1] Meier, R., and Meyer, R. Th., *Schweiz. Med. Wschr.*, 1941, **71**, 1266.

Methods. Adult cats of both sexes were prepared under urethane or DIAL-With-Urethane for carotid cannulation to record arterial pressure by the Anderson[3] manometer. The vagi were severed. The drugs employed, injected intravenously on a basis of milligrams per kilogram, included: epinephrine HCl, priscol HCl, atropine SO_4, ephedrine SO_4 and pituitrin.

In some of our experiments shielded electrodes were attached to either or both sets of splanchnic nerves and the adrenal glands were extirpated or ligated.

The secretory function of the submaxillary gland, as influenced by injection of drugs or faradization of the ipsilateral chorda tympani and cervical sympathetic nerves, was determined after cannulation of Wharton's duct by measuring the salivary flow on a horizontally placed manometer. Pupillary responses were observed and measured directly.

Results. Twenty-four experiments were performed and the most significant findings were:

1. Priscol, 5 mg, without exception, produced a drop in blood pressure of 50% or more; pressure gradually returned to normal within an hour or two. Adrenolysis invariably prevailed for vasoconstrictor neural components since an "epinephrine reversal" was consistently obtained during the hypotension caused by priscol. Sympatholysis of vasoconstrictor components, however, did not prevail with this dose of priscol, although it could be produced readily with a 10 to 15 mg dosage (Fig. 1).

2. Priscol was both adrenolytic and sympatholytic in respect to sympathetic control of salivary secretion. The response to epinephrine was usually diminished or lost before the response to faradization of the cervical sympathetic nerve had been completely abolished. The required dose of priscol varied from 1 to 20 mg for adrenolysis and from 5 to 20 mg for sympatholysis. No consistent correlation could be established between the sympathetic inhibition of salivation and the loss of vasoconstrictor control of blood pressure effected with equal doses of priscol. These dose ranges were greater than those suggested in our preliminary report.[2]

3. Atropine, 0.2 mg, did not interfere with the antisympathetic actions of priscol and neither did pituitrin (Fig. 1).

4. Priscol also inhibited the salivary response that normally is invoked by ephedrine.

5. Priscol initiated salivation, but in only 2 cases and only after high dosage, 12 to 30 mg.

6. The pupil responded well after priscol by dilating in response to administration of epinephrine or to faradization of the cervical sympathetic nerve. In some of our experiments, priscol caused constriction of the pupil; this occurred with a dose of 3 to 5 mg.

Discussion. With these experiments it has been confirmed that priscol possesses the dual antisympathetic action of other known sympathetic-blocking agents, namely, the adrenolytic and sympatholytic actions which characterize such substances. This is most obvious in the responses of the vascular system after priscol: epinephrine reversal precedes nullification of splanchnic vasoconstriction to faradization.

In order to rule out the severe hypotension induced by priscol as the cause of splanchnic nerve paralysis, the pressure was raised by pituitrin (Fig. 1); during this elevation of tension adrenolysis and sympatholysis still prevailed.

Atropine was employed for two reasons: (1) to determine the effect of the loss of cholinergic control of salivation in relation to priscol's antisympathetic actions and, (2) to rule out vagal stimulation of the gut by faradization since vagal components may run through splanchnic nerves, in which case con-

---

[2] Chess, D., and Yonkman, F. F., *Fed. Proc.*, 1945, **4**, 114.

[3] Anderson, F. F., *J. Lab. and Clin. Med.*, 1941, **26**, 1520.

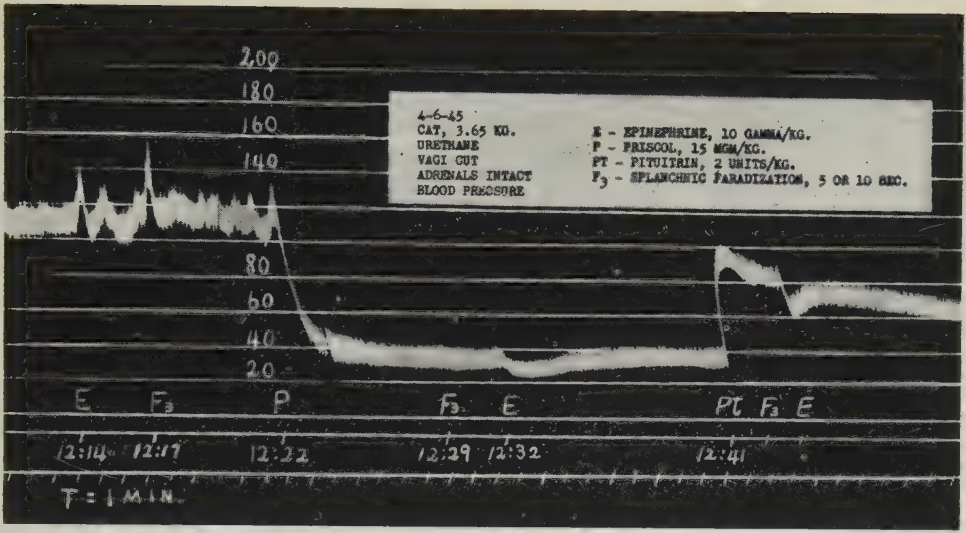


Fig. 1.

traction of the intestinal musculature under vagal influence might account in part for elevating blood pressure; this could be erroneously interpreted as resulting from splanchnic vasoconstriction. The blood pressure, however, was elevated during splanchnic faradization in the atropinized animal and, therefore, such pressure elevation must have been due only to vasoconstrictor stimulation.

In reference to pressure variations, it seemed to make little difference whether the adrenal glands were intact. In almost every instance faradization of the splanchnic nerves resulted in vasoconstriction, as evidenced by hypertension. Rarely, after priscol paralysis of vasoconstrictors did faradization of the splanchnic nerves produce a slight, transient drop in pressure. This may have been due to splanchnic stimulation of the adrenal gland, resulting in the production of epinephrine, which, in the presence of adequate amounts of priscol, would produce a typical "epinephrine reversal." Such a drop in pressure could also have attended the added relaxation of the intestines resulting from faradization of splanchnic inhibitory fibers to the musculature of the intestine. Since this slight pressure drop occurred also in the absence of the adrenals, it was undoubtedly due chiefly to intestinal relaxation attending stimulation of sympathetic inhibitory fibers in the splanchnic nerves.

The fact that adrenergic salivation, as produced by epinephrine, ephedrine, or cervical sympathetic faradization is depressed by priscol indicates that this drug is a general sympathetic depressant, more potent in respect to some functions than to others. Militating against this generalization, however, is the persistence of mydriatic responses to epinephrine and faradization after priscol in the doses employed. Whether higher doses would nullify pupillary responses is problematic; in any event, sufficient evidence is presented to indicate that priscol varies in its adrenolytic and sympatholytic potencies in the cat in respect to different sympathetic functions. Such variance might prevail in other species, including man.

The antisympathetic properties of priscol suggest the use of this drug in Raynaud's disease as recently reported by Lindquist[4] and in other conditions of sympathetic predominance, for instance in hypertension of neurogenic origin as postulated by Heymans[5] where it should act not symptomatically as a depressor but etiologically as an antagonist to excessive adrenergic stimuli.

---

[4] Lindquist, T., *Acta Med. Scand.*, 1943, **113**, 83, abstracted in *J. A. M. A.*, 1945, **127**, 618.

[5] Heymans, C., and Bouckaert, J. J., *C. R. Soc. Biol.*, 1931, **106**, 471.

Summary and Conclusions. Priscol (benzylimidazoline) is sympatholytic as well as adrenolytic in reference to the sympathetic neural control of blood pressure and salivation. Adrenolysis invariably precedes sympatholysis with any dose that is adequate to produce both effects. These pharmacodynamic actions persist during priscol hypotension, pituitrin hypertension and in the presence of atropine.

Although sympathetic depression of salivation and vasoconstriction can be produced with variable doses of priscol, up to 15 mg, they are without sympatholytic or adrenolytic effect upon the cervical sympathetic neural components controlling mydriasis, indicating that a differential sympathetic depression characterizes this antisympathetic agent.

15249

Observations on Cobalt Polycythemia. I. Studies on the Peripheral Blood of Rats.*

ALLAN J. STANLEY, HOWARD C. HOPPS, AND ARTHUR A. HELLBAUM.
From the Departments of Physiology, Pathology, and Pharmacology, University of Oklahoma School of Medicine, Oklahoma City, Okla.

The experimental production of polycythemia by the administration of cobalt has been demonstrated by a number of investigators since its discovery by the Waltners.[1-9] This effect of cobalt has also received application in the treatment of various anemias in both animals and human beings.[10,11]

The mechanism of action of cobalt remains obscure. Several theories have been advanced, however none has been fully agreed upon as a satisfactory explanation by those who have investigated the problem. During the course of some experiments relating to factors influencing various types of anemia, certain new observations concerning the action of cobalt came to our attention.

Male rats of the Sprague-Dawley strain, 4 to 5 months of age, weighing approximately 200 g were given cobaltous chloride, $CoCl_2 \cdot 6(H_2O)$, 0.4% solution, by subcutaneous injection in the back and shoulder region 6 times weekly throughout a period of 8 months. Nine animals, divided into 3 groups of 3 each, received 2.5 mg, 5.0 mg and 10.0 mg per kg of body weight respectively. Other animals of the same age, weight, and sex served as controls. The rats were fed dog chow only (Friskies, Albers Milling Co.)

Erythrocyte, reticulocyte, leucocyte and differential counts as well as hemoglobin determinations were made every 2 weeks. Care was taken to draw only the amount of blood necessary for the determinations, the puncture wound in the tail vein being promptly closed after each bleeding. A Haden-Hausser hemoglobinometer (Improved Clinical Model) was used for the hemoglobin determinations.

At the end of 32 weeks, blood volumes

---

* This work was supported by a grant from the University of Oklahoma School of Medicine.

[1] Waltner, K., and Waltner, K., *Klin. Wchnschr.*, 1929, **8**, 313.

[2] Orten, J. M., Underhill, F. A., Mugrage, E. R., and Lewis, R. C., PROC. SOC. EXP. BIOL. AND MED., 1931, **29**, 174.

[3] Orten, J. M., Underhill, F. A., Mugrage, E. R., and Lewis, R. C., *J. Biol. Chem.*, 1932, **99**, 457.

[4] Davis, J. E., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 96.

[5] Barron, A. G., and Barron, E. S. G., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 407.

[6] Mascherpa, P., *Arch. ital. biol.*, 1930, **82**, 112; *Chem. Abstr.*, 1930, **24**, 3280.

[7] Sutter, J., *C. R. Soc. de biol.*, 1934, **116**, 994.

[8] Stare, F. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1933, **99**, 473.

[9] Davis, J. E., McCullough, A. W., and Rigdon, R. H., *J. Lab. and Clin. Med.*, 1945, **30**, 327.

[10] Kleinberg, W., Gordon, A. S., and Charipper, H. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 119.

[11] Kato, K., *J. Ped.*, 1937, **11**, 385.

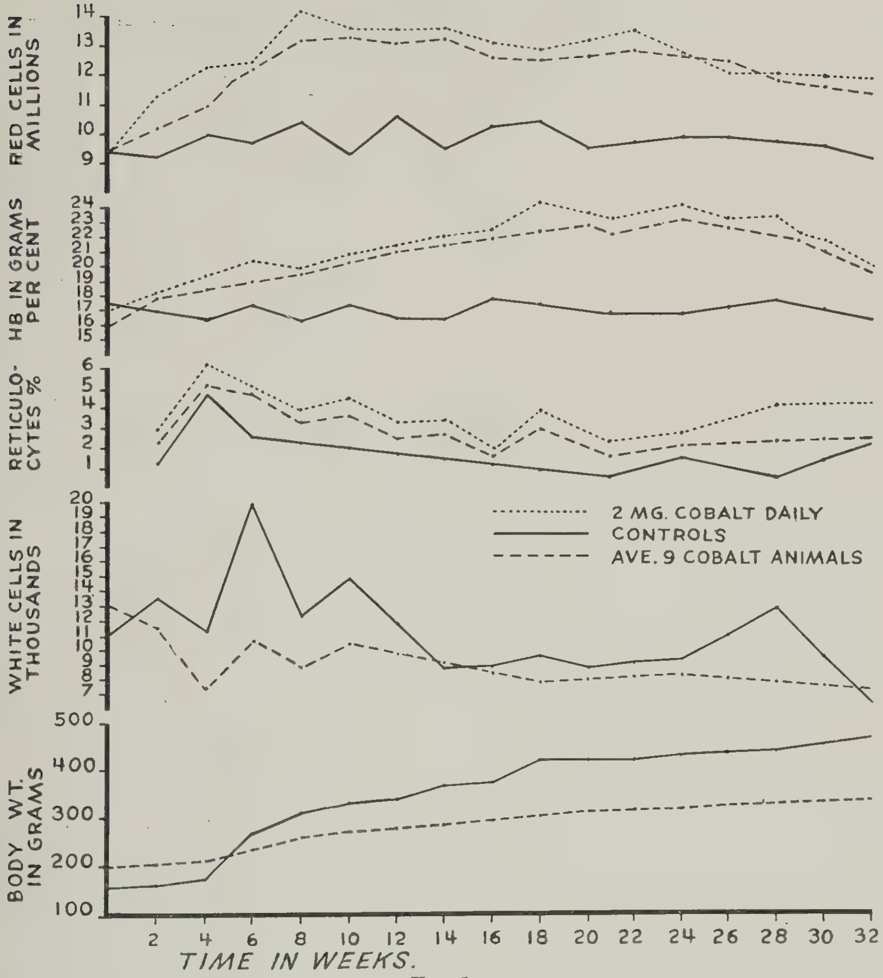


Fig. 1.

The course of polycythemia produced by cobalt in rats. The dotted line in each of the 3 upper charts shows the maximal values in red cells, hemoglobin, and reticulocytes produced by 2 mg of cobaltous chloride daily.

ere determined in the following manner.
he dye T-1824 (Evans blue) in a solution of
.0 mg per ml was injected into a tail vein
amounts of 1.5 mg per kg of body weight.
wenty-five minutes±30 seconds were al-
wed for the dye to mix with the circulat-
g blood. The animals were lightly an-
esthetized with ether for one to 1½ minutes
rior to drawing 2.0 ml of blood by heart
uncture. A precise amount of dry am-
onium and potassium oxalate was used as an
ticoagulant. Samples for blood counts were
ken from the non-oxalated blood while
nears for differential leucocyte and reticulocyte counts were made from the oxalated blood. The mean cell diameters of the red cells were determined by means of a Haden-Hausser erythrocytometer using a fresh solution of 1.4% sodium oxalate as the diluent. Hematocrit determinations were made and the supernatant plasma was used for plasma volume calculation. A Klett-Summerson Photoelectric colorimeter employing a Wratten filter F-29 was used to determine the dye concentration. In order to limit blood loss to a minimum, the plasma of each animal was diluted from 1:4 to 1:7 with saline and centrifuged immediately prior to reading.

TABLE I.
Blood Values of Cobalt-Injected Rats.

Rat No.	Body wt, g	Hematocrit	Blood vol., cc	Blood vol., cc/kg	Plasma vol., cc	Plasma vol., cc/kg	Red cell vol., cc	Red cell vol., cc/kg	Blood/body wt	Red cells/body wt	Plasma/body wt.
1	340	76	44.46	130.8	10.67	31.4	33.79	99.4	13.07	9.94	3.14
2	345	Data lost									
3	360	72	49.51	137.6	13.86	38.6	35.65	99.0	13.76	9.90	3.86
4	264	81	43.00	162.8	8.17	30.9	34.83	131.9	16.28	13.19	3.09
5	343	65	41.60	121.2	14.56	42.4	27.04	78.9	12.12	7.88	4.24
6	338	75	59.24	175.2	14.81	43.8	44.43	131.4	17.52	13.14	4.38
7	296	76	47.84	161.6	11.48	38.7	36.36	122.9	16.16	12.29	3.87
8	332	77	54.04	162.7	12.43	37.4	41.61	125.3	16.27	12.53	3.74
9	321	74	44.56	138.8	11.58	36.0	32.98	102.8	13.88	10.28	3.60
Avg	324	74.5	48.03	148.8	12.19	37.4	35.83	111.4	14.88	11.14	3.74
Controls.											
10	457	43.5	34.12	74.6	19.38	42.4	14.74	32.2	7.46	3.22	4.24
11	487	48	38.00	78.0	19.76	40.5	18.24	37.4	7.79	3.74	4.05
12	315	49	29.6	94.0	15.1	47.9	14.5	46.0	9.40	4.60	4.79
13	312	44	26.4	84.6	14.8	47.5	11.6	37.1	8.46	3.71	4.75
Avg	393	46.1	32.03	82.8	17.26	44.5	14.79	38.2	8.28	3.82	4.45
Difference between controls and experimental animals.											
		28.4	16.00	66.0	5.07	7.1	21.04	73.2	6.60	7.32	.71
% increase or decrease experimental over controls.											
		+61	+50	+80	—29.3	—16	+142	+192	+80	+192	—16

Data derived from hematocrit and plasma volume determinations at the end of 32 weeks of cobalt injections. Rats 1, 2, and 3 received 0.5 mg cobalt daily; rats 4, 5, and 6 received 1.0 mg cobalt daily; rats 7, 8, and 9 received 2.0 mg cobalt daily.

TABLE II.
Data Relative to Shape, Size, and Hemoglobin Content of Red Cells of Rats After Injections of Cobalt.

	Controls	Cobalt	% increase
Mean cell diameter in μ	6.45	6.55	—
Mean red cell volume in μ^3	47.0	66.5	41.0
Mean red cell thickness in μ	1.44	1.97	36.8
Mean red cell hemoglobin in μμg	17.0	17.0	—
Mean red cell hemoglobin conc. in %	36.4	26.5	—27.4

Data relative to the red cells of rats injected with cobalt. The mean corpuscular volume and mean corpuscular thickness are derived from red cell counts, hematocrit determinations and mean cell diameters. The hemoglobin values are calculated from hemoglobin determinations, red cell counts and total red cell mass.

Calibration curves were made on rat plasma containing known amounts of T-1824 and also diluted 1:4 to 1:7. Dilution of rat plasma with as much as 7 parts of saline did not alter the absorption characteristics of T-1824 as determined by the photoelectric colorimeter. At the time the blood volume determinations were made, blood counts also were made using blood taken from a tail vein and from the heart of each animal. The differences between the two counts for each animal were within the tolerated limits of technical error.

Fig. 1 summarizes the data obtained at

regular 2-week intervals. It is apparent that the response of reticulocytes, red cells, and hemoglobin is influenced slightly by the dosage of cobalt but that the average figure for the 3 groups is close to that elicited by the maximum dose of 10.0 mg per kg of body weight. The curves for body weight and leucocyte count similarly show no marked differences as affected by the 3 doses given. While the experimental animals failed to gain as much weight as the controls, no other deleterious effects were noted, which indicates a low systemic toxicity from cobaltous chloride in adult animals in the dosage administered.

Table I presents the numerical data for each animal relative to the blood volume at the end of 32 weeks. The striking increase in total red cell mass and the slight but consistently lower plasma volumes are worthy of note.

Table II contains data derived from cell counts, measurements of the mean cell diameter, hematocrit and hemoglobin content. The cell size, cell volume, cell thickness, absolute hemoglobin content and hemoglobin concentration were calculated according to Wintrobe.[12] Regarding the size, shape and hemoglobin content of erythrocytes from animals with cobalt polycythemia the following facts have come to light as a result of this study: 1. Although the mean cell diameter remains normal the cell volume increases by about one-third, a change which is proportionate to the increase in cell thickness. 2. The absolute amount of hemoglobin per red cell remains unchanged.

The blood volume per kilogram of body weight of these animals compares favorably with that found in the more extreme cases of polycythemia vera in man.[13]

Detailed histopathologic studies are in progress as well as further work on the components of red cells and plasma.

Conclusions. 1. Injections of cobaltous chloride continued over a period of 8 months without apparent serious toxicity has resulted in persistent polycythemia during this period of a degree comparable to that occurring in polycythemia vera in man. 2. The average increases in blood volume and total erythrocyte mass as compared with controls was 80% and 192% respectively, due entirely to increase in erythrocytes since plasma volume decreased 16% on the average. 3. The erythrocyte of polycythemic animals as compared with the normal increased in volume 41% due almost entirely to an increase in the thickness of the cell. The hemoglobin content remains essentially unchanged.

---

[12] Wintrobe, M. M., *Clinical Hematology*, Lea & Febiger, 1942.

[13] Gibson, J. B., 2d, Harris, A. W., and Swigert, V. W., *J. Clin. Invest.*, 1939, **18**, 621.

15250

Influence of Dimethylaminoethylbenzhydryl Ether Hydrochloride Upon Histamine Flare Reactions.

Herbert Elias, and Thomas H. McGavack. (Introduced by Israel S. Kleiner).
From the New York Medical College, Metropolitan Hospital Research Unit, Welfare Island, New York City.

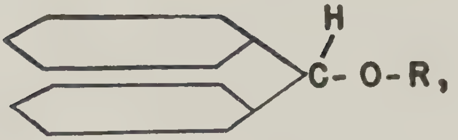
The alleviation of histamine induced anaphylaxis in guinea pigs has been described following the use of a number of benzhydryl alkamine ethers of the general formula, where R represents a carbon chain carrying a primary, secondary or, in most cases, a tertiary nitrogen atom.[1,2,3] In one of the

---

[1] Loew, E. R., Kaiser, M. E., and Moore, V., *J. Pharm. and Exp. Therap.*, 1945, **83**, 120.

[2] Loew, E. R., and Kaiser, M. E., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 235.

[3] Sharp, E. A., personal communication.



most potent of these preparations, the R grouping is beta-dimethylaminoethyl.[1,2]

The present experiments have been conducted to determine the degree to which histamine flare reactions in the skin can be influenced by the oral administration of dimethylaminoethyl benzhydryl ether hydrochloride* to human subjects.

Methods and Materials. Human beings who were not known to have any allergic disease or other condition associated with a recognizable disturbance of the autonomic nervous system were subjected to histamine flare tests before, during, and after the oral administration of varied amounts of dimethylaminoethyl benzhydryl ether hydrochloride.

To examine skin sensitivity to histamine, a drop of 1% aqueous histamine solution and a drop of physiological saline for control purposes were each applied to the volar surface of the forearm. A slight depression of the skin under each drop was then made by pressure from a sterilized ordinary pin, care being taken not to cause any bleeding. After 5 minutes, the drops were gently wiped away, and the size and shape of the wheal and erythema were outlined with ink. This outline was accurately transferred for permanent record by the use of transparent paper and india ink. At the end of 10 minutes a similar reading and recording of the wheal and erythema was made. The size of the wheal and of the erythema were approximated by multiplying the diameters at right angles by each other. The values thus obtained in each subject in the fore period were arbitrarily taken as 100%, and all subsequent values computed as fractions or multiples thereof.

Results. The data (Table I) reveal that a daily dose of 150 mg of dimethylaminoethylbenzhydryl ether hydrochloride was capable of a definite reduction in the sensitivity of the skin to histamine, and that 300 mg daily might effect a maximum response if sufficiently long continued. A diminution in both wheal and erythema has been observed after 3 days of treatment with 150 mg daily. A complete disappearance of both wheal and erythema has been noted in some cases after 10 days of treatment with 300 mg. The continued administration of the drug, at least for periods up to 18 weeks apparently does not alter the striking anti-histamine action. As a rule a maximum effect can be attained with a dose of 300 mg daily, although this may not be reached quite so promptly as when 400 mg are given.

When the drug is discontinued, the response to histamine is accentuated as compared with the control period. In one instance, the "release" phase was associated with a flare and wheal that were approximately 500% of the original. In every case, this phasic effect is seen, but the response to histamine usually returns to normal within a few days after dimethylaminoethyl benzhydryl ether hydrochloride has been stopped. In no previously normal subject was this increased response to histamine associated with any clinical allergic manifestation.

Discussion. Undoubtedly the anti-histamine action of dimethylaminoethylbenzhydryl ether hydrochloride accounts for the clinical success attendant upon its application to several types of skin condition.[4,5] The high therapeutic index, the failure for tolerance

---

* Generous supplies of this material under the name, Benadryl, have been made available through the courtesy of Dr. E. A. Sharp, of Parke, Davis & Co., Detroit, Mich.

[4] Curtis, A. C., and Owens, B. B., *Univ. Hosp. Bull.*, Ann Arbor, 1945, **11**, 25.

[5] O'Leary, P. A., and Farber, E. M., *Proc. Staff Meetings of the Mayo Clinic*, 1945, **20**, 429.

[6] McElin, T. W., and Horton, B. T., *Proc. Staff Meetings of the Mayo Clinic*, 1945, **20**, 417.

[7] Koelsche, G. A., Prickman, L. E., and Carryer, H. M., *Proc. Staff Meetings of the Mayo Clinic*, 1945, **20**, 432.

[8] Williams, H. L., *Proc. Staff Meetings of the Mayo Clinic*, 1945, **20**, 434.

[9] Logan, G. B., *Proc. Staff Meetings of the Mayo Clinic*, 1945, **20**, 436.

[10] McGavack, T. H., Elias, H., and Boyd, L. J., *J. Lab. and Clin. Med.*, in press.

TABLE I.
Erythema and Wheal Reactions to the Intracutaneous Administration of Dimethylaminoethyl Benzhydryl Ether Hydrochloride.

No. subjects	Dosage (mg/daily)	Duration treatment (weeks)	Degree of Reaction (% of control)‡			
			Erythema		Wheal	
			5 min.	10 min.	5 min.	10 min.
10	0	—	100	100	100	100
5	150	0.6	105	63	63	75
5	300	2.0	4	37	3	7
3	400	2.3	24	33	4	8
6	—	0.5†	138	155	185	168
2*	—	1.0†	493	300	104	104

* Both of these patients had received treatment for more than 11 weeks, and at the 400 mg dosage level for not less than 2 weeks.

† Length of time since treatment was discontinued.

‡ Figures represent averages for the number of subjects indicated.

to develop, even on long continued administration, and the clinical results thus far attained,[4-10] warrant further extensive clinical trial of the drug in a wide variety of allergic conditions.

Summary. Dimethylaminoethylbenzhydryl ether hydrochloride orally administered in sufficient amounts is capable of completely suppressing the sensitivity of the skin to histamine, at least as expressed by the wheal and flare reaction. In limited trials, this effect appears to have widespread clinical application.

15251

The Reputed Antipyretic Action of Camphor.*

ELDON M. BOYD, AND KATHLEEN G. W. SEYMOUR.
From the Department of Pharmacology, Queen's University, Kingston, Canada

Many current textbooks of pharmacology contain statements to the effect that camphor is antipyretic and useful in the symptomatic treatment of fevers. Thus for example, in Sollmann's treatise[1] we find, "Camphor Spirit is a household remedy in colds, bronchitis, etc. It is thought to be somewhat expectorant, diaphoretic and antipyretic (Binz, 1875 and 1877)." In older books, published 100 years or more ago, less cautious statements are made, for there the virtues of camphor as an antipyretic are panegyrised. Camphor was first used in China and brought to Europe by the Arabians.[2] Its introduction as an antipyretic seems to have been based upon at least two properties, first that it has some antidiarrheic activity and secondly that spirit of camphor, applied to the skin, has a cooling effect. There has been no recent work done to check these ancient conceptions of the antipyretic value of camphor.

In the work described in this report, some 500 animals, including albino rats, guinea pigs, rabbits and cats, were rendered febrile by the intramuscular injection of peptone (B.D.H.) in a dose of 1 g per kilo body

---

* This research was aided financially by a grant from the Committee of Scientific Research, Queen's University. A report of the work was presented at the annual meeting of the American Society for Pharmacology and Experimental Therapeutics, Inc., Atlantic City, March, 1946.

[1] Sollmann, T., *A Manual of Pharmacology,* 6th ed., Saunders, London, 1942.

[2] Flückiger, F. A., and Hanbury, D., *Pharmacographia,* Macmillan, London, 1879.

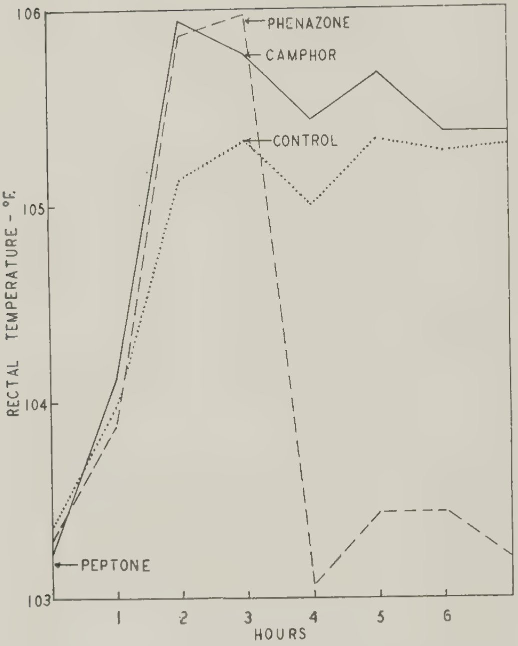


Fig. 1.

The effect of phenazone and of camphor upon peptone-induced fever in rabbits.

weight. In the first experiment, 66 rabbits were divided into 6 groups. Rabbits in 3 of the groups were injected with peptone while rabbits in the other 3 groups received no peptone. Rectal temperatures were taken before the injection of peptone and at hourly intervals thereafter, the latter in all groups of animals. Three hours after the injection of peptone, when fever was at its height, rabbits in one group were given an intramuscular injection of camphor in oil at a dose of 100 mg per kilo body weight; at the same time the second group of febrile rabbits received phenazone B.P., (antipyrine, U.S.P.) at a dose of 200 mg per kilo body weight; the third group of febrile rabbits received no further treatment and acted as controls. The remaining 3 groups of afebrile rabbits, which received no peptone, were treated in a corresponding manner at the end of the third hour of the experiment, *i.e.* one group received phenazone, the second camphor and the third no further treatment.

There was no appreciable change, over the period of 7 hours of the experiment, in the mean rectal temperature of rabbits receiving no peptone, no camphor and no phenazone. Neither did phenazone nor camphor have any significant effect upon the mean rectal temperature of afebrile rabbits. There were distinct changes in rectal temperature of rabbits injected with peptone and the means of these have been plotted in Fig. 1. The control febrile rabbits developed a mean rectal temperature which varied between 105° F. and 106° F. The injection of phenazone rapidly reduced the febrile temperature to normal. The injection of camphor, however, had little effect upon febrile temperature.

This experiment demonstrated that camphor, in the dose used, was not antipyretic under conditions in which phenazone had a marked antipyretic effect. There was the possibility, however, that camphor might have been antipyretic in smaller or larger doses than that used. To investigate this, a second experiment was performed in which 6 groups of 12 rabbits each were given peptone and then respectively, 0, 1, 10, 50, 100, and 500 mg per kilo body weight of camphor dissolved in oil and injected intramuscularly. In this entire series of animals, camphor had no consistent effect upon the febrile temperature.

Thus far, our experiments proved that camphor was not antipyretic when injected into rabbits with a fever induced by injection of peptone. We next considered the possibility that camphor might be antipyretic in other species of animals. Accordingly, the technique used in the second experiment, just described, was applied to 72 albino rats, 66 guinea pigs and 80 cats, each divided into 6 groups, injected with peptone and the respective doses of camphor. The 500 mg per kilo dose of camphor produced convulsions in cats and was discontinued after 5 trials. In no instance was there any evidence that camphor had any appreciable antipyretic activity.

In these 3 experiments, camphor was not found to be antipyretic in animals with a fever induced by injecting peptone. There was the possibility that it might be antipyretic towards fever induced by pyrogens other than peptone. From time to time, we had available animals with a natural fever due, usually, to infection and abscess formation at the point where peptone had been injected. This group of animals included 9 rabbits, 6 guinea pigs, 8 albino rats and 6 cats. We made no systematic study of these but injected them with various doses of camphor in oil. In the entire group of 29 animals, 24 or 83% showed no change or increased fever after injecting camphor, in 4 animals the temperature fell toward normal and 1 animal died.

Finally, we considered the possibility that camphor given by stomach tube in the form of Spirit of Camphor, B.P., might be antipyretic. Six groups of 13 rabbits each were injected with peptone and 3 hours later given respectively 0, 0.01, 0.1, 0.5, 1.0, and 5.0 ml of Spirit of Camphor by stomach tube, washed down with water. Again camphor failed to exhibit any antipyretic activity.

These 5 experiments convinced us that camphor has little or no antipyretic activity and that statements to the effect that the internal use of camphor is "supposed" or "considered" to have some antipyretic value should be deleted from textbooks, thus in some measure, even though small, lightening the burden of information which the medical student, physician and pharmacologist have to learn.

Summary. Camphor was administered parenterally and by stomach tube, in a wide range of doses, to some 500 animals, including albino rats, guinea pigs, rabbits and cats with a fever induced by previous injection of peptone and, in a few instances, fever from infection. Under these conditions, phenazone was found to be antipyretic in rabbits, but no evidence was obtained that camphor possessed antipyretic properties, at least to any marked degree.

15252

Blood Chemical Changes Following Intravenous Administration of a Casein Hydrolysate to Human Subjects.*

William S. Hoffman, Donald D. Kozoll,† and Bess Osgood.

From the Hektoen Institute, and the Departments of Surgery, Cook County Hospital and Northwestern University Medical School, Chicago, Ill.

To determine the criteria for the proper administration of Parenamine,‡ one of the commercially available casein hydrolysates which are used parenterally, and as a part of an investigation of its clinical effectiveness, a study was made of some of the blood chemical changes that occurred when this preparation was injected intravenously. The clinical studies have been reported elsewhere.[1]

Forty-five g of Parenamine (equivalent to 6 g of nitrogen) dissolved in 1000 cc of distilled water were injected intravenously into 30 hospital control subjects at a commonly used clinical speed of 300 to 350 cc per hour. The injections were made in the morning, breakfast having been withheld. Blood samples were drawn at the beginning, middle and end of injections and at 1 and 2 hours after the injection was completed. Plasma amino acid nitrogen concentrations were de-

---

* Aided by a grant from Frederick Stearns and Company, Detroit, Mich.

† Abbott Fellow in Surgery, Northwestern University Medical School.

‡ Kindly furnished by Frederick Stearns and Company, Detroit, Mich. Parenamine is an acid hydrolysate of casein, fortified with 1% d, l-tryptophane. It is put up in 15% solutions in bottles containing 100 cc. The method of preparation of Parenamine is essentially that described by Sahyun.[7] An analysis of the distribution of the amino acids in the preparation has been reported by Block and Bolling.[8]

[1] Kozoll, Donald D., Hoffman, William S., and Meyer, Karl A., *Arch. Surg.*, 1945, **51**, 59.

TABLE I.

Blood Chemical Changes Following Intravenous Injection of 45 G of Casein Hydrolysate.

Blood Constituent Analyzed	No. of Cases	Mean Levels During and After Injection					Avg. Max. Change	Modal Time of Max. Change
		At Start of Inj.	Middle of Inj.	End of Inj.	1 Hr. After	2 Hr. After		
Amino Acid N, mg N per 100 cc Plasma	30	5.1 ±1.5* 2.5-8.4†	9.4 ±1.4 6.1-11.2†	9.2 ±1.2 7.2-11.2†	7.0 ±1.2 4.5-9.0†	6.0 ±1.1 4.0-8.0†	+4.9 ±1.7	Middle of Inj.
Urea N, mg, N per 100 cc blood	19	11.5	13.6	15.7	17.4	17.3	+6.6	1 Hr. after Inj.
Inorganic Phosphate, mg P per 100 cc Plasma	18	3.6	3.1	3.5	3.9	4.0	—0.5	Middle of Inj.
CO_2 Combining Power, cc per 100 cc Plasma	13	63	62	61	62	64	—4	End of Inj.

* Numbers after ± represent standard deviation.
† Range.

termined by the naphthoquinone method adapted to the photoelectric colorimeter by one of us (W.S.H.),[2] inorganic phosphate photoelectrically by Fiske and Subbarow's method,[3] urea by the method of Hoffman and Osgood[4] and CO_2 combining power by the method of Van Slyke,[5] and glucose by the method of Hoffman.[6]

The results are summarized in Table I. The initial plasma amino acid N levels ranged between 2.5 and 8.4 mg per 100 cc averaging 5.1 with a standard deviation of ±1.5. These values are slightly higher than those obtained with the ninhydrin method.[9] The amino acid N levels rose during the injection reaching a maximum in the majority of cases in the middle of the injection, in the remainder at the end of the injection. Thus the rate of removal of amino acids from the blood stream eventually became faster than the 15 g per hour of injection. The average maximal rise was 4.9 mg per 100 cc. At the end of 2 hours the amino acid N concentration had returned almost to the base level. The amino acid curves on the whole agreed with those found by Landesman and Weinstein[10] for intravenous injection of Amigen.

The blood urea N concentration began to rise slowly during the injection reaching a maximal value in 11 cases at 1 hour after the injection, and in 8 cases at 2 hours after the injection. The average maximal urea N concentration was 17.4 mg per 100 cc. The highest maximal concentration was 27.1 mg

---

[2] Hoffman, William S., *Am. J. Clin. Path.*, 1945, **15**, 57.

[3] Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, 1925, **66**, 375.

[4] Hoffman, William S., and Osgood, Bess, *J. Lab. and Clin. Med.*, 1940, **25**, 862.

[5] Van Slyke, D. D., *J. Biol. Chem.*, 1917, **30**, 347.

[6] Hoffman, William S., *J. Biol. Chem.*, 1937, **120**, 51.

[7] Sahyun, M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 14.

[8] Block, R. J., and Bolling, D., *Am. J. Pharm.*, 1944, **116**, 368.

[9] Woodruff, C. W., and Man, E. B., *J. Biol. Chem.*, 1945, **157**, 93.

[10] Landesman, R., and Weinstein, V. A., *S. G. and O.*, 1945, **75**, 300.

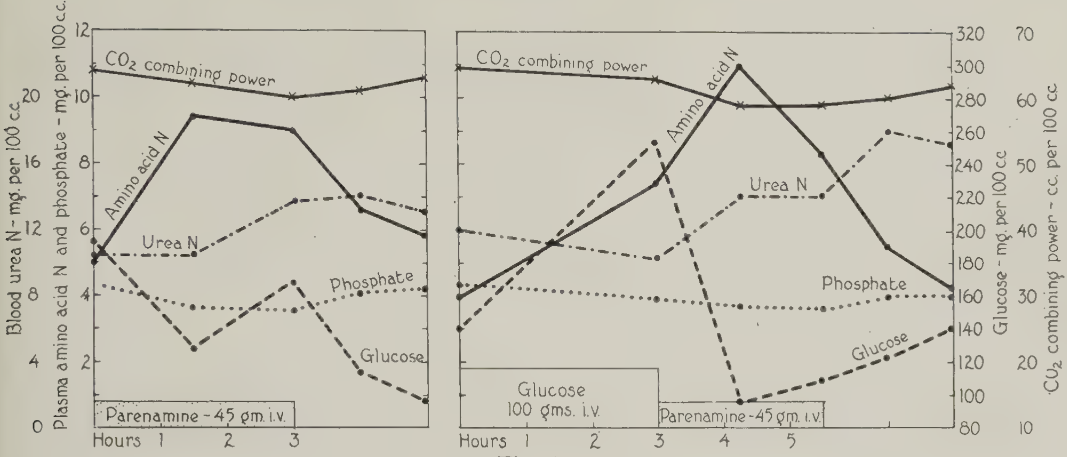


Fig. 1.

per 100 cc; this was in a patient whose initial level was 18.3 mg per 100 cc and who therefore may have had an early renal insufficiency. With one other exception, the remaining subjects showed maximal urea concentrations within the limits of normal such as might be found in patients on a high protein diet. Urea clearance determinations made in the successive intervals in 8 cases were too variable to indicate any direction of change, but at least they gave no clear evidence that the rise in blood urea concentration was due to a diminished rate of renal excretion.

A rather surprising finding was the drop in serum inorganic phosphate. This decline was small, about 0.5 mg per 100 cc, but was statistically significant. It occurred in all experiments, and the t value[11] for statistical significance of the difference between the average initial level and that at the middle of the injection was 2.9, a value above the 2.5 required to make it improbable that the decline was fortuitous. The maximal decline occurred at the midpoint at the same time that the amino acids were most rapidly leaving the blood stream.

Since Parenamine is furnished as an unneutralized mixture of amino acids having a pH of about 4.2, it is important to determine whether injections of this product produce any appreciable changes in CO_2 combining power. The results summarized in Table I indicate that the fall in CO_2 combining power is negligible. The range of maximal change was from +3 to —11 cc per 100 cc with an average fall of 4 cc per 100 cc. In 8 out of 13 cases, the fall occurred at the end of the injection. A return to the initial levels had occurred at 2 hours after the completion of the injection.

In 30 experiments listed in Table I, vomiting occurred in 2 cases at or near the end of the injection. In both cases the amino acid N levels were over 11 mg per 100 cc. Two other patients complained of nausea at a time when the amino acid levels were over 10 mg per 100 cc. In all 4 cases the rate of injection was somewhat faster than the average of 3 hours for the 1000 cc. In 5 additional cases not listed, injections were purposely made at fast rates ranging from 90 to 120 minutes for the 1000 cc. In 2 of these experiments, the maximal amino acid N concentration was over 12 mg per 100 cc and were associated with a sense of fullness, nausea, and vomiting. Thus it appears that if the plasma amino acid N concentration rises considerably above 10 mg per 100 cc, there may be symptoms produced comparable to those in acute overeating. It may be that the increase in concentration of a certain amino acid, such as glutamic or aspartic acid, is actually responsible for the symptoms.

---

[11] Snedecor, G. W., *Statistical Methods Applied to Experiments in Agriculture and Biology, Collegiate Press, Inc.*, Ames, Iowa, 1937.

Amino acid tolerance curves obtained after oral ingestion of 45 g of Parenamine (in another study from this laboratory[12]) were similar to those after intravenous injection except that the average maximal rise was only 3.4 mg per 100 cc, the amino acid N level seldom rising above 10 mg per 100 cc.

Since glucose is frequently administered intravenously along with amino acids, it was found desirable to study the blood chemical changes when a liter of 4.5% solution was injected following the intravenous administration of a liter of 5% glucose. There was no appreciable effect upon the various curves. One such experiment is shown in Fig. 1. The glucose curve fell promptly to normal following the injection. The serum phosphate level, depressed by the glucose injection was further lowered by the amino acid injection, reaching in one case the low level of 1.5 mg per 100 cc. The rise to normal levels was rapid.

Summary. Injection of 45 g of Parenamine intravenously at slow speeds produced an average plasma amino acid N rise of +4.9 mg per 100 cc during the injection with a return to normal in 2 hours. Rises to levels above 10 mg per 100 cc produced by rapid injection may be associated with nausea and vomiting. A progressive though slight rise in blood urea takes place during and after the injection, accompanied by increased urea excretion. Serum inorganic phosphate is lowered during the injection and returns to normal by the end of the injection. Only a negligible drop in CO_2 combining power occurred during the injection. Intravenously injected glucose did not alter the curves produced by the amino acid injection.

---

[12] Hoffman, William S., and Dyniewicz, H., *Gastroenterology*, in press.

15253

Is Normal Human Urine Toxic?*

Bernhard Zondek, and Rivka Black.

From the Gynecologic-Obstetric Department, Rothschild Hadassah University Hospital, and the Hormone Research Laboratory, Hebrew University, Jerusalem, Palestine.

Much investigation has been devoted to the question of whether normal urine is toxic. It has been suggested that the increase of a toxic urine constituent is associated with disturbance of renal function (e.g. uremia, eclampsia). Aschheim and Zondek, however, in developing the hormonal pregnancy test concluded that human urine is non-toxic for the mouse, since injections of very large doses of human urine are tolerated by this animal.

Rachmilewitz[1] on the basis of experiments with fibroblast cultures *in vitro* concluded that human urine, native as well as dialyzed, contains, even in a dilution of 1:50, a powerful toxic substance which inhibits the growth and brings about the degeneration of cells. Uremic and normal urine were found to contain this toxic substance in about equal amounts.[1] It is, however, uncertain whether results of experiments carried out *in vitro* can be applied also *in vivo*, especially where toxicity is in question, since a cell (fibroblast) *in vitro* may lack detoxication potentialities which characterize the intact organism. Many substances whose toxic effect *in vitro* is notorious (e.g. para-chloro-xylenol[2] and many others) are completely non-toxic *in vivo*. Although dialyzed human urine inhibits the growth of fibroblasts *in vitro*[1] we found that it has no toxic action on a surviving frog

---

* Partially aided by a grant from the Ella Sachs-Plotz Foundation.

[1] Rachmilewitz, M., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 497; *Arch. Int. Med.*, 1941, **67**, 1132.

[2] Zondek, B., *Nature*, 1942, **149**, 334; *J. Urol.*, 1942, **78**, 747.

TABLE I.
Amounts of Normal Human Urine Tolerated Without Toxic Reaction by Different Animal Species.

Animal	Wt.	cc of urine and distribution of dose	Route of injection	Urine / body wt.
mice	7.5 g	6 X 0.4 = 2.4	s. c.	$\frac{2.4}{7.5} = \frac{1}{3}$
,,	10 ,,	30 (as 3 cc of dialyzed and conc. urine)	,,	$\frac{30}{10} = 3$
rats	30 ,,	90 X 1 = 90	,,	$\frac{90}{30} = 3$
,,	100 ,,	30 X 10 = 300	,,	$\frac{300}{100} = 3$
rabbit	1500 ,,	9 X 10 = 90	i. v.	
,,	1000 ,,	250 (as 20 cc of dialyzed and conc. urine)	,,	$\frac{250}{1000} = \frac{1}{4}$
dog	15 kg	160	,,	

heart (Straub preparation). Native human urine added to the surviving frog heart caused immediate cessation of the heart beat (hypertonic salt content). Urine was dialyzed and after 5-fold concentration, rendered isotonic with NaCl. This preparation was entirely without toxic effect on the surviving heart. The experiment shows that though dialyzed urine may contain a constituent which is *in vitro* toxic to fibroblasts, such urine even in 5-fold concentration *in vivo* is non-toxic not only in respect to the intact organism but even in respect to a surviving organ.

In further experiments toleration of normal urine by different species was studied:

A dog weighing 15 kg received intravenously 160 cc of native human urine with no undesirable by-effect.

An adult rabbit received intravenous injections of 90 cc of native urine in 9 doses of 10 cc each given at intervals of 1 hour. The animal showed no toxic response. Rabbits weighing 1 kg were injected intravenously with 20 cc of dialyzed urine concentrates, the equivalents of 100-250 cc of native urine. These injections elicited no toxic after-effect.

Infantile rats (30 g) were given daily injections of 1 cc of native human urine for 3 months, *i.e.*, a total of 90 cc. The onset of sexual maturity was not affected by this protracted treatment with urine. Rats weighing 100 g received daily an injection of 10 cc of native human urine for 1 month, a total of 300 cc of urine. Nevertheless, a pregnant animal in this group produced a normal litter.

Urines were dialyzed and concentrated up to 10-fold *in vacuo*. Three cc samples of the concentrates were injected into mice weighing 10 g in 3 injections during 8 hours. The mice showed no toxic reaction to these injections, the equivalent of up to 3 times the body weight.

Our experiments show that animals tolerate normal human urine about as well as they do physiological saline (Table I).

Urine has been administered for therapeutic purposes to man in recent years both intramuscularly, rectally and orally. Ovarian dysfunction has been treated by intramuscular injections of native pregnancy urine, which is rich in gonadotropic and estrogenic hormone. In these cases, daily injections of 10-20 cc of pregnancy urine over a period of several weeks elicited no toxic effect. Russian authors have recommended treatment of severe uterine bleeding during puberty by a rectal drip infusion of several liters of native pregnancy urine. The experience of our clinic has corroborated the non-toxicity of this treatment. It may be mentioned also that Atcheson,[3] in order to save penicillin, administered urine of penicillin-treated patients to cases of chronic gonorrhea via a duodenal tube with good results.

The conclusion follows from the above findings that normal human urine is non-toxic *in vivo* for animals or man.[†]

Does Ether "Detoxicate" Urine? At the time of development of the pregnancy test, it was found that 7% of urine samples were unsuitable for examination by the method proposed at that time, because their injection into the mouse caused death, mostly within 24 hours. Zondek[4] reported that "toxic" urines of this type become innocuous to mice if they are first shaken with ether. This observation has since been confirmed by numerous workers. Experiments which are reported below show that the ether treatment is effective not because it extracts a specific toxic constituent but because it frees the urine from microorganisms which contaminate it during the passage over the vulva.

1. An ether extract of "toxic" urine was taken up in oil and injected into mice. It proved completely non-toxic.

Chemical restitution of the extracted urine by the re-addition of the ether extract failed to restitute toxicity.

Addition of the ether extract to non-toxic urine from another source did not render the latter toxic.

2. Ether had a bacteriostatic or a bactericidal effect on the bacterial flora of the urine. When ether-treated urine which had been allowed to stand at 37° C for 24 hours remained sterile, it proved also to be non-toxic. On the other hand, when development of bacteria occurred in the extracted urine sample during the period of the incubation, the original toxicity of the urine was found to have reverted. Proteus bacteria could be isolated from the heart blood of mice which had died following an injection of a "toxic" urine sample. Injection into the mouse of 1 cc of a broth culture of the isolated Proteus strain caused death.

3. In all cases, "toxic" urine could be rendered innocuous to the mouse by boiling.

4. Urine was taken by means of a catheter from patients whose urine had proved toxic when it had been taken in the ordinary way. It proved to be non-toxic when it was taken by catheter.

Conclusion. Tolerance tests for urine on a surviving organ (frog heart), and on different species of laboratory animals as well as clinical findings in man prove that human urine is not toxic. The delayed fatal effects which follow the injection of certain urine specimens (7%) into animals are due to infections. Ether treatment renders such urine specimens innocuous not because it removes a toxic substance from the urine but because it is bacteriostatic or bactericidal.

Our thanks are due to Dr. Daniela Weber for bacteriological tests.

---

[3] Atcheson, D. W., *Military Surgeon*, 1944, **95**, 58.

[†] Urine may, of course, contain toxic materials, either drugs or hormones, after medication, but such urines cannot be looked upon as normal urines.

[4] Zondek, B., *Klin. Wschr.*, 1930, **9**, 964.

15254

A Chick Embryo Technic for Intravenous and Chemotherapeutic Studies.*

HENRY F. LEE, ABRAM B. STAVITSKY, AND MARGARET P. LEE.
(Introduced by Werner Henle.)

From the School of Medicine, Department of Pediatrics, University of Pennsylvania and the Children's Hospital of Philadelphia.

The chick embryo has become increasingly valuable in investigative work and in the production of certain biologic materials. Up to the present time no less than 20 viruses have been propagated by its use. Many of the bacteria, certain rickettsias, leptospira icterohemorrhagica, and a growing number of types of tissue cells, both benign and malignant, have been studied in the embryo or upon its membranes. Several recent studies have dealt with chemotherapeutic testing as applied to experimentally infected embryos. None of these papers has given detailed quantitative information concerning levels attained by therapeutic agents in the blood and extraembryonic fluids.[1-5] In the course of the above types of studies many technics have been devised.

Rous and Murphy were the first to use the chorio-allantoic membrane for the study of transplanted tumor fragments. They were also the first to use the chick embryo in the study of an infectious agent, the virus of the Rous Sarcoma.[6]

The majority of studies involving the exposed chorio-allantoic membrane as a site of inoculation have made use of a method of opening eggs first described by Clark.[7] Woodruff and Goodpasture modified Clark's technic.[8] Later Goodpasture and Buddingh described improvements and their technic, with slight variations, has been used by many laboratories.[9] The technic described by Burnet involving the production of an artificial air sac has also found wide use for inoculation of the chorio-allantoic membrane and for infection by the amniotic route.[10] Taylor and Chialvo reported a simplification of Burnet's method which they found particularly valuable for intraamniotic injections.[11] A method for intravenous inoculation of the embryo was described by Eichorn.[12] Many modifications of the above technics have been devised.

Each of the technics mentioned is satisfactory for certain purposes. However in the course of experiments concerned with the chemotherapy of bacterial infections produced in chick embryos by intravenous inoculation,† it became apparent that no method previously described was entirely satisfactory. A method was required that would permit intravenous inoculation of the embryo as well as accurate injection into or aspiration from any or all of the extraembryonic sacs at the same or at separate times. It was also necessary that the method permit later sampling of the blood. The method here reported permits all of the above procedures as well as placement of

---

* These studies were supported by a grant from the Heyden Chemical Company.

[1] Bang, F. B., and Bang, B., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 527.

[2] Morrow, G., and Berry, G. P., *J. Bact.*, 1938, **36**, 280.

[3] Ransmeier, J. C., *J. Infect. Dis.*, 1943, **72**, 77.

[4] Weil, A. J., and Gall, L. S., *J. Infect. Dis.*, 1941, **69**, 97.

[5] Parker, R. F., and Diefendorf, H. W., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 351.

[6] Rous, P., and Murphy, J. B., *J. A. M. A.*, 1911, **56**, 74.

[7] Clark, E. R., *Science*, 1920, **51**, 371.

[8] Woodruff, A. M., and Goodpasture, E. W., *Am. J. Path.*, 1931, **7**, 209.

[9] Goodpasture, E. W., and Buddingh, G. J., *Am. J. Hyg.*, 1935, **21**, 319.

[10] Burnet, F. M., *Brit. J. Exp. Path.*, 1940, **21**, 147.

[11] Taylor, R. M., and Chialvo, R. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 328.

[12] Eichorn, E. A., *Science*, 1940, **92**, 245.

† To be reported separately.

TABLE I.
Average Water Loss (g) 10th Through 20th Day of Incubation Each Figure Is Average of 12 Eggs.

	Unopened eggs	Opened, membrane exposed, single layer Scotch-tape	Ditto double layer	Ditto triple layer
Wt. loss in g	4.0	7.7	5.1	4.6

TABLE II.
Volume of Blood (cc) Obtained from Embryos of Various Ages.

	Age in Days						
Embryo No.	10	11	12	13	15	17	18
1	.08	.20	.23	.26	.30	.39	.70
2	.05	.18		.29	.32	.42	.70
3	.10	.23		.31	.33	.40	.50
4	.09	.20		.31	.29	.33	.60
5	.12	.28		.20	.36	.43	.65
6	.12	.10	.24	.34	.36	.43	.75
Avg.	.09	.19		.28	.32	.39	.65

material upon the exposed chorio-allantoic membrane.

Technic. Eggs of the selected incubation-age are candled and the air sac margin is marked with a pencil. For intravenous work the embryo should be of at least 10 days incubation. The shell along the air sac margin is wiped off with 70% alcohol. When many eggs are handled a squat, wide-mouthed bottle filled with 70% alcohol with a pad of cotton over its mouth facilitates this step. The cotton is allowed to project into the alcohol-bearing cotton. The shell over the air sac is removed by cutting just distal to the pencil line with a thin carborundum disc rotating at high speed. The motor is fixed in a vise and the egg rotated in contact with the cutting disc. With a little practice this "decapping" procedure can be done very rapidly. It is possible to remove the shell cap entirely or to leave it attached by partially cut shell so that it serves temporarily as a cover against air-borne dust. If the cap has been left attached it is easily removed with cover-slip forceps. The egg is inverted and tapped lightly to remove shell dust from the surface of the reflected portion of the shell membrane. The rest of the work requires a satisfactory egg-holder. A low wide-mouthed bottle with its open end covered with cotton moistened with 70% alcohol is ideal for this purpose. The cotton supports the egg firmly but permits instant adjustment of position. Good lighting is also important. A light source in front of the operator has been found best, provided that a piece of Polaroid is placed between the light source and the egg. The Polaroid serves to eliminate glare from the moist membrane surfaces.

As recommended by Burnet in his description of a side-window technic about 0.1 cc of sterile saline solution is placed over a slight nick made in the shell membrane overlying the chorio-allantoic membrane. The nick is made with the fine needle which deposits the saline. The saline solution serves to initiate separation of the shell membrane from the underlying chorio-allantoic membrane. In the presence of this saline the shell membrane may then be gently picked up with fine-pointed forceps and stripped off with no resultant capillary bleeding. The entire surface of the chorio-allantoic membrane lying under the air sac may be exposed or as much of this area as is desired for the particular work being done. Injection intravenously, into the amniotic fluid, into the allantoic fluid, or into the yolk sac, may then be made under full visibility. Blood or extra-embryonic fluids may be collected or materials may be deposited upon the surface

TABLE III.
Survivals After Opening Shell, Exposing Chorio-allantoic Membrane and Sealing with Scotch Tape for the Remainder of Incubation. Three Separate Groups of Eggs.

Days age	10	15	17	20
Untouched controls	18	18	18	18
Opened group 1	18	18	18	18
” ” 2	18	18	16	16
” ” 3	18	18	18	18

TABLE III-A
Same as Table III Plus Injection of .1 cc of Saline Into the Yolk, Into the Amniotic Fluid, and Into the Allantoic Fluid.

Days age	10	15	17	20
Untouched controls	18	18	18	18
Opened. No injection	18	17	16	16
” Injected	18	16	15	15

TABLE IV.
Intravenous Injection.
Survival Rates with .05 cc Saline as Inoculum. Injection Time Approximately 5 Seconds.
All Control Groups Opened, Membrane Exposed, Sealed.

	Age in Days					
	10	11	12	15	18	20
Controls	18	18	18	18	18	18
Large vessels, no hemostasis	48	21	21	18	17	17
Controls	18		16	16	16	16
Fine vessels selected	18		12	11	9	9
Controls	18		18	18	18	18
Fine vessels selected	18		15	14	13	13
Controls		18	18	18	18	18
Fine vessels selected		18		15	15	15
Controls		18		16	16	16
Fine vessels selected		18		14	12	12
Vessel entered with needle but no injection		18		15	14	14

of the membrane. The collection of blood is difficult before the ninth day of incubation.

The open end of the egg is then sealed with 3 layers of transparent Scotch tape. The triple layer is used since it permits the closest approximation to normal water-loss during the remainder of the incubation period as determined by loss of weight. (Table I.) Substances may later be applied to the chorio-allantoic membrane by simply passing a fine needle through the Scotch tape. At any time the tape may be partially or completely removed for further work. The tape should be applied in such lengths that it extends down over the egg for approximately ½ inch.

Intravenous Injection. Embryos show individual variations of the pattern of veins in the exposed chorio-allantoic membrane. Usually there are several venous branches available for injection. In an occasional egg no suitable vessels are present in this area. The usual situation is to find one large allantoic vein with two or more tributaries. Very small vessels may be entered with a No. 27 needle provided its point is perfectly sharp and its shaft smooth. It is preferable to use the tributaries and not the main vessel since less bleeding results upon withdrawing the needle. When a large vessel is used it is possible to control the bleeding by the use of very small "serrefine" hemostats. The open jaws of these small spring-activated hemostats are slipped onto the vessel just as the needle is withdrawn and the hemostat is held in place for 15-20 seconds before removal. Hemostasis is complete. An increase in mortality results from this clamping procedure but this increase is not regularly predictable. This step is not necessary if the small vessels are chosen for injection.

TABLE V.

Intravenous Injection of Saline. Effect of Volume and Rate of Injection.

Days old	10	11	12	13	14	15	16	17	18	19	20
A. Effect of Rate of Injection											
Controls	18			16			16			16	16
.05 cc in 2 sec. approx.	18			9			9			9	9
.05 cc in 5 sec. approx.	18			12			11			9	9
.05 cc in 60 sec. approx.	18			11			11			8	8
B. Effect of Volume of Injection											
Controls	18			18			18			18	
.05 cc in 5 sec. approx.	18			15			14			13	
.1 cc in 5 sec. approx.	18			9			4			3	
.2 cc in 5 sec. approx.	18			3			1			1	

TABLE VI

Compound	No. of embryos	Dose in mg per 100 g of egg wt.	% survival* after 9 days
Promin	18	60	0
	18	50	50
	18	40	80
	18	30	100
Sulfathiazole	18	20	50
	18	15	84
	18	10	100
Promizole	12	5	0
	12	3	75
	12	2	92
	12	1	100
3,3′-dimethyl-4,4′-diaminodiphenyl sulfone	12	5	0
	12	2	75
	12	1	100
3-methyl-4,4′-diaminodiphenyl sulfone	12	5	0
	12	2	59
	12	1	81
	12	.5	100
Diamino diphenyl sulfone	12	5	0
	12	2.5	0
	12	1.5	46
	12	1.	83
	12	.5	92

In all cases above compounds were injected into yolk sac on the 10th day of incubation. Regardless of route of administration it was found that *comparative* toxicity remained the same.

* All figures are corrected for mortality of controls.

Quarter cc tuberculin syringes and ½ inch No. 27 needles have been found most suitable.‡

Collection of Blood Samples. Bleeding is accomplished by one of two methods. If it is desired to obtain more than one sample from an embryo one of the branches of the allantoic vein must be used. A citrated 1.5 cc syringe and a No. 25 needle is suitable. When withdrawing blood after entering the vein, very gentle intermittent traction on the syringe plunger is employed. When it is desirable to obtain as large a blood specimen as possible, it is best to use the large main trunk of the allantoic vein as it proceeds toward the embryo in a fold of membrane. An avascular area of the chorio-allantoic membrane is selected. With two pairs of fine pointed forceps the membrane is picked

---

‡ Results obtained by the use of this method for intravenous inoculation with suspensions of tubercle bacilli will be the subject of a separate report.

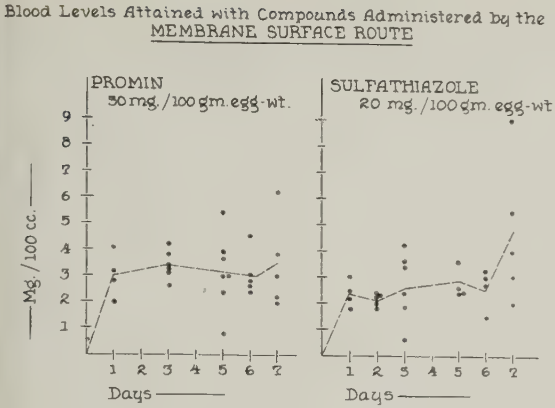


Fig. 1.

up and torn in this area. The allantoic vein is grasped with forceps and a No. 25 needle is inserted. The needle is advanced into the vein for approximately one-quarter inch. The forceps may then be removed and blood gently aspirated. The amount of blood obtainable from the embryos of various ages is shown in Table II.

Chick embryos are notably resistant to accidental infection, and it has been the experience of others that it is possible to work in an open room without any special precaution against air-borne contamination. The studies here recorded were carried out as aseptically as possible under a hood. Occasional contamination occurs under the best of conditions.

Advantages of the Technic. The principal advantages of the technic may be summarized as follows:

1. Opening and sealing of eggs is relatively rapid.

2. Adequate exposure is obtained for selective or combined intravenous, intra-yolk, intra-amniotic or intra-allantoic injection or aspiration.

3. All structures are clearly visible, thereby assuring accuracy of placement of injected materials.

4. The same egg may be used for later bleeding and/or sampling of the extra-embryonic fluids.

5. Embryo and membranes remain visible during incubation. Candling is unnecessary since death of the embryo is immediately apparent through the large transparent window.

6. The feasibility of selecting very small allantoic venous tributaries for intravenous injection minimizes bleeding.

Mortality from Procedure. When using embryos of 10 days incubation or more the mortality resulting from opening the shell and exposing the chorio-allantoic membrane is negligible. (Table III). Injection of sterile saline solution into the amniotic, allantoic, or yolk sac does not increase the mortality. (Table III, A).

Following intravenous injection of sterile saline solution the mortality rises sharply when using 10-day embryos. With the use of 11-day embryos the survival rate improves somewhat. (Table IV). Volume of injection, within limits, is more important than rate of injection. (Table V). In addition to the data given in these tables, a series of experiments involving controls receiving intravenous saline solution has demonstrated a uniformly higher survival rate when 11-day-old embryos are used as compared to 10-day-old embryos. This age factor is important principally in regard to intravenous work. For other types of work, survivals are excellent with embryos 5 days of age and up.

Quantitative Studies of Compounds Administered to Chick Embryos. Although the chick embryo is being used for pilot testing of compounds for possible chemotherapeutic value, there are few data available concerning the distribution of such compounds after their placement within the egg. It has been shown that certain sulfonamides are rapidly distributed through the egg but reports are chiefly qualitative.[2,3,4] Depending upon the route of inoculation and upon the conditions of a given experiment, information concerning levels attained in yolk, amniotic, and allantoic fluid may be of value. Experimental infection of the embryo itself may be produced by intravenous inoculation. Under such circumstances a knowledge of blood levels reached by chemotherapeutic compounds makes possible a more definitive interpretation of results.

Two compounds were chosen for quantitative studies. One, sulfathiazole (2-(p-amino-

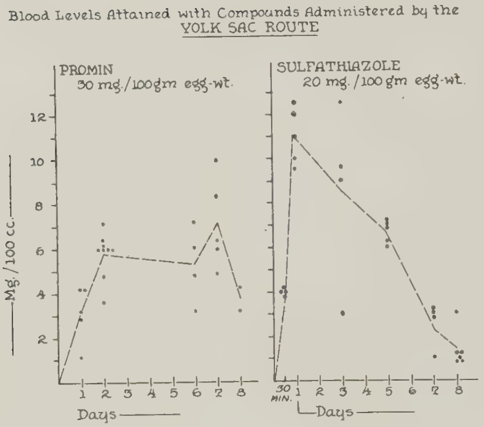


Fig. 2.

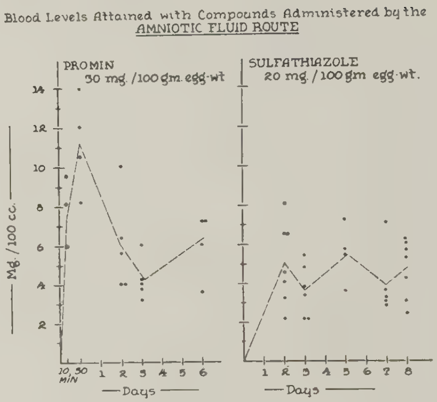


Fig. 3.

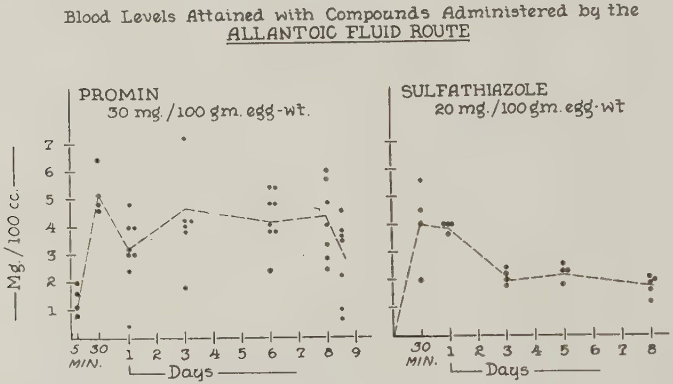


Fig. 4.

benzenesulphonamido)thiazole), is a relatively insoluble sulfonamide while the other, Promin, (sodium p-p′ diaminodiphenylsulfone N-N′ didextrose sulfonate), is a highly soluble sulfone. In addition a few streptomycin assays have been completed.

Methods. The technic described above was used throughout. The compounds were administered as a 5% solution in the case of Promin or as a 5% suspension of microcrystals in the case of sulfathiazole. Dosage was computed in terms of milligrams per 100 g of egg weight. Although the weight of the eggs used varied from 48 to 64 g, the dosage was computed on the basis of the uniform egg weight of 55 g. This introduces an error in dosage not exceeding 15%. All possible routes of administration were tested.

Toxicity studies were carried out first. Maximum tolerated doses by the yolk sac route were determined. The findings for promin, sulfathiazole, and 4 other compounds are given in Table VI. For these toxicity studies the compounds were injected into the yolk sac on the 10th day of incubation. Toxicity was determined in terms of embryo survival to the 20th day of incubation. The figures are corrected for the mortality of controls from the same group of eggs. The controls were handled exactly as the test embryos except that no material was injected. Eighteen embryos were used for each dosage group for the promin and sulfathiazole, while in the case of the other compounds 12 were used for each group.

On the basis of these toxicity studies dosages of the 2 compounds were selected for quantitative studies. The following graphs reveal the results obtained. All figures represent milligrams per 100 cc of fluid or blood. Although the emphasis was placed upon blood levels, a number of determinations in the

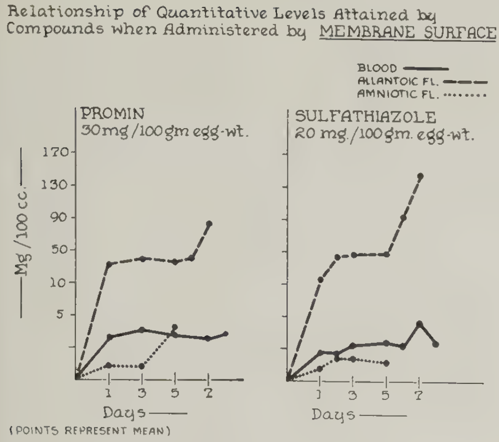


Fig. 5.

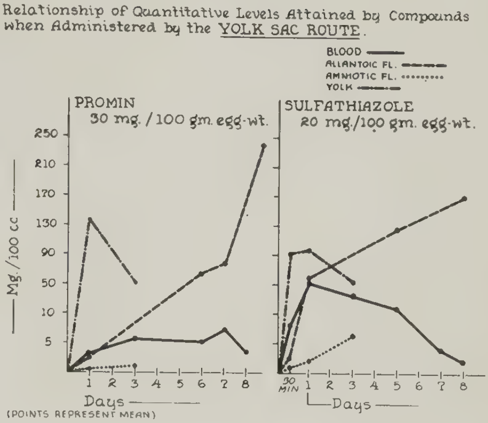


Fig. 6.

other fluids were done and suffice to give an indication of the distribution of the compounds. A total of 850 quantitative determinations were made. Studies with low doses and with toxic doses are omitted from the tables. Determinations were made by the Proom modification of the Marshall method.[13]

In all cases the figures represent single specimens from individual embryos and not serial specimens from the same embryo. It is possible, however, when such data is needed, to obtain at least 3 blood specimens at intervals from an individual embryo.

The few streptomycin determinations that have been completed indicate that this substance is present in the blood in a concentration of 6 to 10 units per cc of blood 12 hours after the placement of 1000 units on the surface of the chorio-allantoic membrane. Higher levels over longer periods are maintained in the allantoic fluid.

Discussion. Despite its limitations, which must be clearly recognized, the chick embryo is a valuable laboratory aid for screening procedures in chemotherapeutic research. It presents both simple (membrane mesenchyme) and highly complex tissues for study. Because of its immunologic status it is adaptable to certain bacteriologic problems wherein tissues are needed which are, so far as has been ascertained, free from antibody and sensitization phenomena. Within the egg one may study infection in the presence of living parenchymatous organs with their enzyme systems and metabolic processes intact. Naturally occurring infection of any sort in the chick embryo is a rare occurrence. A large number of embryos may be maintained in scarcely more space than is required for tissue culture studies. It is believed that the technic here presented may serve to facilitate such investigations as may make use of the chick embryo.

Summary. A chick embryo technic is described which possesses certain advantages over reported methods. The technic is applicable to many types of investigation. Quantitative data are presented on the distribution of sulfathiazole and promin within the fertile egg.§

---

[13] Proom, H., *Lancet,* 1938, **1**, 260.

§ This work was carried out in laboratory space provided by The Henry Phipps Institute of the University of Pennsylvania.

15255 P

Effects of Electro-Convulsive Shocks on Daily Activity of Albino Rats in Revolving Drums.

CALVIN P. STONE.

From the Department of Psychology, Stanford University.

Exploratory activity and rate of locomotion of rats in mazes and other learning situations falls off sharply if they are subjected to a series of electro-convulsive shocks.[1,2] Their performances are characterized by delays in starting, frequent pauses, and lethargic movements. These observations suggest the desirability of further study of the voluntary activity of rats in non-learning situations such, for example, as the Slonaker-type of revolving drums. A preliminary study was undertaken with 6 adult females.

For the first 3 weeks no convulsive shocks were administered. During this time the animals had established characteristic trends of daily activity which, although variable from day to day, nevertheless seemed sufficiently stable to enable one to detect sudden deviations of large magnitude. At the beginning of the fourth week each rat received a convulsive shock daily for 5 days. This was followed by 5 days without shock, 5 days with daily shock, and so on, for 3 alternating periods of shock and no-shock, plus 2 additional post-shock periods.

To cause the convulsion, a 70-volt alternating current was applied to the rat's scalp over the forebrain for approximately .2 second through alligator-clip electrodes. The electrodes were fastened on the dorsal scalp approximately 6 mm apart and 1 cm behind an imaginary line between the eyes. The scalp was moistened with a salt solution where the clips were attached. Each convulsion had the characteristic features of a grand mal seizure, starting with vigorous tonic contractions and passing into the clonic phase after about 10 seconds. The total seizure lasted between 20 and 30 seconds. Immediately after the convulsion was over most of the animals were hyposensitive to mild external stimuli for from 2 to 3 minutes. Then followed a short period of hypersensitivity, ushered in by startle reactions or milling about the cage when lightly stimulated by the experimenter or a cage mate. Occasionally during the hypersensitive stage an animal would attempt to bite the hand of the experimenter. The animals were replaced in the activity drum immediately after their shocks where they were free to sleep or to rotate the drum. Only one animal (No. 1) regularly revolved the drum soon after the convulsion, this occurring while she was in the hypersensitive stage.

In Fig. 1 are given the mean numbers of revolutions made by each animal during nine successive 5-day periods. The characteristic level of each rat's activity just prior to the first series of convulsions is shown in period 1. In periods 2 to 7 appear the means for alternating periods of shock and no-shock. Periods 8 and 9 show the gradual return to the pre-shock level of activity on the part of most of the rats.

As is apparent, the rats were much less active during the shock periods than during the no-shock periods. Reduction began in the first 24 hours after a convulsive shock and disappeared during the second day beyond the last shock. Rat No. 4, a relatively inactive animal, was an exception to this generalization, however. It ceased running almost altogether after its first period of electro-shocks and did not again become active during the 2 weeks beyond the last convulsion.

In this preliminary study we did not attempt to correlate changes in activity with possible disturbances of the estrus cycles. Nevertheless, the suddenness with which activity was depressed by the first convulsion clearly suggests that the primary reduction was due to some direct effect of the convul-

---

[1] Stainbrook, E. J., *J. Psychol.*, 1942, **13**, 337.

[2] Siegel, P. S., *J. Compar. Psychol.*, 1943, **36**, 61.

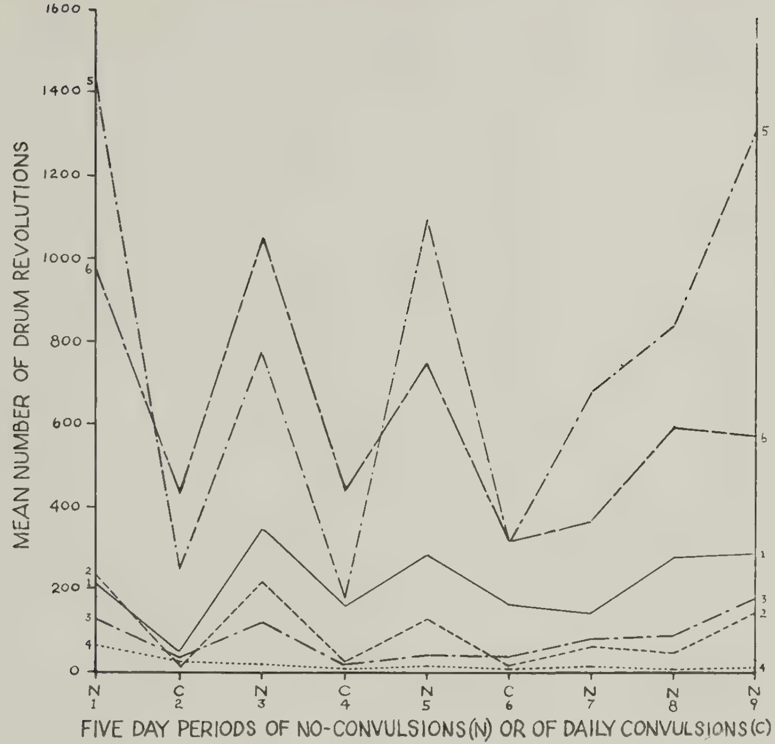


Fig. 1.

Activity records of 6 female rats. In periods 1, 3, 5, 7, 8, and 9 the usual conditions for rats in activity drums prevailed; whereas, in periods 2, 4, and 6 the rats received one electro-convulsive shock each day. Each rat's number is given on the left and right ordinate.

sive shock. Eventually, however, a disturbance of estrus might have been a contributing factor. Further studies on this and other aspects of the problem are in progress.

Conclusion. Electro-convulsive shocks in 6 female rats greatly reduced the amount of their voluntary activity for a short period of time. The major change appears within the first 24 hours and disappears within 48 hours after the last convulsive shock is administered. Within a 2-week period following their last convulsion the majority of the animals closely approximated their pre-shock levels of daily activity.

15256

Changes in the Hydrogen Ion Concentration of Healing Fractures.

Orvar Swenson and C. Lloyd Claff. (Introduced by E. C. Cutler.)

From the Laboratory for Surgical Research, Harvard Medical School, Boston, Mass.

The repair process involved in fracture healing is of great interest both from theoretical and practical standpoints. Repair of bone differs from soft tissue repair, in that the process involves calcification and ossification. The morphological changes in fracture repair are well established and have recently been reviewed by Urist and Mc-

TABLE I.
pH Changes in Healing Fractures.

Time after fracture			2½ Hrs.	24 Hrs.	3 Days	5 Days	7 Days	9 Days	11 Days	13 Days	15 Days
pH	Hematoma	220-43	7.18	7.07	7.19	7.22	7.29	7.36	7.32	7.47	7.53
,,	,,	217-43	7.42	7.33	7.26	7.28	7.29	7.51			
,,	,,	235-43	7.34	7.25	7.37	7.42	7.52				
,,	,,	215-43	7.28	7.25	7.29	7.24	7.51	7.46			
,,	,,	309-43	7.35	7.08	7.24	7.31	7.41				
,,	,,	333-43	7.36	7.36	7.25	7.30	7.41	7.54			
,,	,,	3-44	7.15	7.13	7.30	7.34	7.44	7.54			
,,	,,	127-43	7.30	6.90	7.02	7.23	7.42	7.60			

Lean.[1,2,3] Less complete biochemical knowledge of fracture repair is available. Clinicians are interested in the process of calcification and ossification, as they are at a loss at times to explain nonunion of fractures or to alter the clinical course of this complication without extensive surgical procedures.

Detection of any postulated biochemical disturbance in calcification in nonunion presents an almost insurmountable problem. First of all, little is known of the biochemistry of normal fracture repair. Most investigations of fracture healing have been morphological studies. Correlation of the biochemical changes with the morphological changes would be interesting and instructive. Analyses of tissue in the region of healing fractures have been made. However, in the early stages of repair there is only a liquid hematoma at the fracture site and tissue analysis is impossible. The presence of the hematoma is fortunate in one respect in that it is comparatively simple to draw out fluid and subject it to chemical analysis. While the hematoma does not represent accurately the biochemical processes in early fracture repair, it probably reflects these factors sufficiently to yield reliable information concerning changes in concentration of various substances as preparations for calcification take place. Assuming that this is true, aspiration of the hematoma and analysis of the fluid is a relatively simple technic for biochemical study of fracture repair. A major problem is the small volume of hematoma fluid present at the site of fracture necessitating the use of microchemical methods.

Determination of the hydrogen ion activity of the hematoma at fracture has been made by Murray,[4] Sterling,[5] and Greune.[6] Technical difficulties seriously limited the number of determinations that were made. Murray and Sterling generally agree, both reporting a pH on the acid side initially, with subsequent change to the alkaline side. In several experiments Greune found a tendency of the fracture site to remain on the acid side. No mention is made of any evidence of infection in these fractures. It was our experience in all instances that persistent acidity at the fracture was accompanied by infection. A desire to determine the timing and extent of pH changes during fracture repair prompted these experiments.

Methods. The first problem was to produce a fracture with a hematoma of adequate size, which remained fluid sufficiently long to permit repeated aspirations for pH determinations. Numerous trials established that the best method to produce such a fracture in a canine femur was to elevate a full thickness spicule of bone and excise a portion of the vastus lateralis muscle, thus providing room for an ample hematoma. Heparin was used in some hematomas in the hope of maintaining a more prolonged fluid state. This technic was of little avail and heparin was not used in the later experiments.

Aspiration of the hematoma was done with 16 gauge needles, having multiple perfora-

---

[1] Urist, M. R., and McLean, F. C., *J. Bone and Joint Surg.*, 1941, **23**, 1.

[2] Urist, M. R., and McLean, F. C., *J. Bone and Joint Surg.*, 1941, **23**, 283.

[3] Urist, M. R., *J. Bone and Joint Surg.*, 1942, **24**, 47.

[4] Murray, C. R., *Indust. Med.*, 1941, **10**, 171.

[5] Stirling, R. I., *Tr. Med. Chir. Soc. Edinburgh*, pp. 203-228, 1931-1932; in *Edinburgh M. J.*, Dec. 1932.

[6] Greune, H., *Deutsche Ztschr. f. Chir.*, 1931, **203**, 324.

tions of the wall near the beveled end. Only rarely did infection of the fracture follow. Most infections seemed to be caused by contamination at operation as the signs of infection appeared early. Late infections attributable to repeated aspirations were rare. Many of the fractures made were unsuitable for study, as the amount of hematoma fluid was insufficient to yield fluid by repeated aspirations for the 7 to 15 days necessary for calcification, demonstrable in a biopsy of the fracture site. Small hematoma and infection made many of the fractures produced unsuitable for study.

Aspiration of the hematoma was carried out 2½ hours after the fracture was produced, again 24 hours later, then every 48 hours until fluid was unobtainable. pH determinations were made in duplicate by means of a micro-glass electrode, designed by one of us—(C. L. C.), capable of making readings on as little as 0.008 ml of fluid. Biopsies were taken at various intervals to correlate the morphology with the pH changes, and to check specifically for beginning calcification by the Kossa staining technic.

Results. The first aspirations, performed 2½ hours after the fracture was produced, yielded a bloody fluid with a pH more acid than that of the venous blood. In a series of 8 fractures, (2½ hours after the fracture was produced) the average pH of the fluid was 7.30, and 24 hours later, the pH in the 8 fractures averaged 7.18. No analysis was carried out to try to determine the substances responsible for the local acidity.

A fracture becomes the center of an intense acute non-infectious inflammatory reaction and, according to Menken,[8] the pH initially in such a reaction should be definitely acid. Breakdown of tissue, damaged at the time of fracture, with more or less isolation of the site of injury from the general circulation, contributes to the local acidity.

As repeated aspirations were performed, at 48-hour intervals, the fluid gradually changed to the alkaline side. In the 8 fractures the average pH at the end of the experiment was 7.52. The shift to the alkaline side, as repair takes place, is difficult to explain. Certainly as the site of injury is vascularized, the local acidity is neutralized. However, this does not explain the peculiar local change to the alkaline side.

The end of each of the 8 experiments was determined by inability to aspirate sufficient fluid for pH determinations and varied from 7 to 15 days. Biopsy of the fracture site was carried out at the termination of each experiment and in 6 out of 8 there was calcification demonstrable by the Kossa staining technic. The material from the remaining two experiments showed questionable calcification. Six other fractures were biopsied from 3 to 5 days after fracture when the pH of the hematoma was still on the acid side or about the same as the venous blood and in none of these was it possible to demonstrate calcification.

The rôle of the hydrogen ion concentration in calcification and ossification is difficult to ascertain. A major rôle has been ascribed to it by the exponents of a theory of calcification.[4,5] This postulates that there is initially a high concentration of calcium ions at the fracture site, due to the increased solubility of the bone calcium salts in the acid hematoma fluid. Actually no measurements of calcium have been reported confirming this theory. As the calcium salts are less soluble in an alkaline solution, it is further postulated that precipitation of calcium takes place as the hematoma at the fracture site becomes alkaline and repair progresses. Certainly such changes in the pH do take place in the hematoma. However, calcification is probably a more complicated mechanism than this theory sets forth. These changes in pH values may be results rather than the controlling factor in calcification.

A persistently low pH at the fracture site invariably indicated infection. It was possible to detect an infected fracture by the persistence of low pH readings before local signs of infection developed. As long as local infection persisted, it was impossible to

---

[7] Claff, C. L., and Swenson, O., *J. Biol. Chem.*, 1944, **152**, 519.

[8] Menkin, V., *Am. J. Path.*, 1934, **10**, 193.

demonstrate calcification until the inflammation subsided.

Conclusions. 1. In the dog, there is an initial local acidity in the hematoma resulting from a fracture, followed by a change to alkalinity beyond the value in the venous blood.

2. Biopsy of the fracture site after the fluid became alkaline, at the end of the experiment, showed calcification in 6 out of 8 cases.

3. Infection at the site of fracture produced a persistent local acidity.

15257

Granulocytopenia in Rats Given Thiourea and Thyroxin. The Therapeutic Effect of *L. casei* Factor.

FLOYD S. DAFT, ARTHUR KORNBERG, L. L. ASHBURN, AND W. H. SEBRELL.

From the Division of Physiology and the Pathology Laboratory, National Institute of Health, U. S. Public Health Service, Bethesda, Md.

The effect of thiourea and its derivatives in depressing the functional activity of the thyroid gland in experimental animals and in man is well known and the literature pertaining to the discovery of this phenomenon has been repeatedly reviewed.[1,2] In the first report on the clinical use of thiourea and thiouracil in the treatment of thyrotoxicosis[1] there was noted the development of agranulocytosis in one patient. A number of additional cases of leucopenia, granulocytopenia or agranulocytosis following the use of these drugs have been described. The importance of finding a method for preventing the development of these blood dyscrasias is obvious. It appeared that there might be an analogous situation in the occurrence of granulocytopenia in rats fed sulfonamides in purified diets[3,4] and the correction of this dyscrasia with *L. casei* factor.[5] It was decided, therefore, to study the effect on rats of administering thiourea in a similar purified diet with and without the simultaneous administration of thyroxin or thyroid powder. If granulocytopenia developed it would then be possible to test the therapeutic value of *L. casei* factor in these animals.

Anemia without granulocytopenia has been encountered in many of the animals receiving thiourea without thyroxin or thyroid powder. Granulocytopenia without anemia has occurred in high incidence in animals receiving both thiourea and thyroxin or thyroid powder and the granulocytopenic animals have been treated successfully with *L. casei* factor.

Experimental. The basal (control) diet used in these investigations consisted of leached and alcohol-extracted casein 18%, Crisco 8%, modified Osborne and Mendel salt mixture[3] 4%, $FeSO_4.7H_2O$ 0.18%, $CuSO_4.5H_2O$ 0.02% and anhydrous dextrose (Merck's U.S.P.) 69.8%. Into each 100 g of diet were incorporated 1 mg of thiamine hydrochloride, 2 mg of riboflavin, 2 mg of calcium pantothenate, 1 mg of pyridoxine hydrochloride and 200 mg of choline chloride. Each rat received a supplement twice weekly of 0.25 cc of corn oil containing 2000 units of vitamin A and 200 units of vitamin D (Natola). The experimental diets differed only in that specified ingredients were substituted for equivalent amounts of dextrose. Diet No. 985 contained 1% of thiourea; diet No. 1053 contained 1% of thiourea and

---

[1] Astwood, E. B., *J. Am. Med. Assn.*, 1943, **122**, 78.

[2] Mixner, J. P., Reineke, E. P., and Turner, C. W., *Endocrinology*, 1944, **34**, 168.

[3] Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L., *Pub. Health Rep.*, 1942, **57**, 1559.

[4] Kornberg, A., Daft, F. S., and Sebrell, W. H., *Science*, 1943, **98**, 20.

[5] Daft, F. S., and Sebrell, W. H., *Pub. Health Rep.*, 1943, **58**, 1542.

TABLE I.
Development of Blood Dyscrasias in Rats Given Thiourea in Purified Diets With and Without the Simultaneous Administration of Thyroxin or Thyroid Powder.

Diet	No. of rats	No. with granulocytopenia[*]	No. with leucopenia[†]	No. with anemia[‡]
Basal control	21	0[§]	0[§]	0
Containing thiourea (No. 985)	28	1	7	14
Basal diet and thyroxin	16	1	0	0
Diet containing thiourea (No. 985) and thyroxin	16	10	10	1
Diet containing thiourea and thyroid powder (No. 1053)	30	18	20	2

[*] 300 or fewer polymorphonuclear granulocytes per cu mm.
[†] 4000 or fewer total leucocytes per cu mm.
[‡] Hematocrit value of 35 volumes % or less.
[§] White cell counts were obtained on only 9 of the 21 control animals.

0.25% of thyroid powder (Armour U.S.P.).

The study consisted of 2 parts; (a) a series of experiments designed to study the development of blood dyscrasias under various conditions and (b) a test of the value of *L. casei* factor in correcting granulocytopenia. For each experiment in the first part of the study weanling male albino rats of the Osborne and Mendel strain were divided into 2 (or 4) groups on the basis of weight and litter. One group was given the basal diet and another the experimental diet containing thiourea (No. 985); when 4 groups were employed the 2 additional ones received these identical diets and in addition were given thyroxin injections. The thyroxin (crystalline, Squibb) was administered subcutaneously 6 times weekly at a level of 1 μg per gram of body weight. In the second part of the study, diet No. 1053, containing thiourea and thyroid powder, was given to all animals throughout the entire experiment. A number of the rats which became granulocytopenic were treated with *L. casei* factor, diet No. 1053 being continued.

At various times, total white blood cell counts, polymorphonuclear granulocyte counts and hematocrit determinations were made on the tail blood of these animals by technics which have been described.[6]

For this report, rats with counts of no more than 300 polymorphonuclear granulocytes per cu mm were considered to be granulocytopenic, those with counts of 4000 or fewer total white cells per cu mm were considered to be leucopenic, and those with hematocrit values of 35 volumes % or less were considered to be anemic. The incidence of granulocytopenia, leucopenia and anemia in the animals subjected to the different experimental conditions is given in Table I. The lowest values observed have been used in the preparation of this summary.

Of 28 animals which were given thiourea but no thyroxin or thyroid powder, only 1 became granulocytopenic while 7 became leucopenic and 14, or 50%, became anemic. Eight of these 14 had hematocrit values of 30 volumes % or less. Results at hand suggest that the anemia cannot be prevented by the inclusion of *L. casei* factor or a crude liver fraction in the diet but the data are not sufficiently extensive to permit a definite conclusion. Animals which received thiourea but also received thyroxin injections or thyroid powder in the diet showed a low incidence of anemia (3 of 46) but a high incidence of granulocytopenia (28 of 46) and leucopenia (30 of 46). Control animals which received no thiourea showed almost no blood dyscrasias (1 of 37) whether or not they were given thyroxin injections.

As a rule, the rats developed the blood dyscrasias after only a few weeks on the experimental diets. The anemia usually appeared somewhat earlier than the granulocytopenia. As noted above 8 rats given the

---

[6] Daft, F. S., Kornberg, A., Ashburn, L. L., and Sebrell, W. H., *Pub. Health Rep.*, 1945, **60**, 1201.

TABLE II
Treatment of 12* Granulocytopenic Rats with *L. casei* Factor (100 μg Per Rat Per Day) for 4 and 10 Days.

	Total white blood cells per cu mm		Total polymorphonuclear granulocytes per cu mm	
	Avg.	Range	Avg.	Range
Before treatment	4630	1300-8850	140	50-300
After 4 days	5470	1650-9250	630*	50-1100*
" 10 "	7920	2950-13,800	2030	800-4100

* A total of 14 animals were treated. One died on the 7th day of treatment and one responded sufficiently well in 4 days that treatment was discontinued. For the sake of uniformity the data for these 2 animals are not included in the table. The animal which responded in 4 days did so on 2 occasions; once from 200 up to 6700 and again from 250 up to 1400 polymorphonuclear granulocytes per cu mm.

thiourea-containing diet (No. 985) became rather severely anemic (hematocrit values of 30 volumes % or less). This degree of anemia developed in these 8 animals usually after about 1 month on experiment (22 to 33 days for 7 animals, average 26 days; 181 days for the eighth animal). The 28 rats which became granulocytopenic while receiving thiourea plus thyroxin or thyroid powder developed this dyscrasia after 25 to 104 days on experiment (25 to 55 days for 25 animals, average 38 days; 75, 81 and 104 days, respectively, for the other animals).

Granulocytopenic rats fed diet No. 1053, containing thiourea and thyroid powder, were used in tests of the therapeutic value of *L. casei* factor.* In preliminary trials it was found that treatment of such animals for 4 days with 25 μg of *L. casei* factor daily did not routinely correct the dyscrasia. The therapeutic dose was increased to 100 μg daily but as indicated in Table II the response even to this amount was not spectacular. Only 2 of 14 animals reached a level of polymorphonuclear granulocytes of 1000 cells per cu mm in 4 days. Treatment was continued in 12 of these animals for 6 additional days and at the end of this time 10 of the 12 animals had 1000 or more granulocytes per cu mm of circulating blood; the remaining 2 had levels of 800 and 950 cells per cu mm respectively.

Pathologic Findings. The heart, lung, liver, kidney, spleen, adrenal and bone marrow of 26 rats fed thiourea were sectioned and examined microscopically. Of these organs only the thyroid glands, adrenal glands, bone marrow and spleen showed significant lesions. The thyroid hyperplasia and colloid depletion found have been previously described. Grossly the adrenal glands did not appear to be enlarged but were cherry red in color. Microscopically, the pathologic alterations present in these glands were hyperemia, often of extreme degree, hemorrhage, necrosis and lipid depletion. The hyperemia and hemorrhage, while commonly present in the inner cortex (reticularis), were not limited to this area. In a few adrenals, hyperemia or hemorrhage or both were present in the outer half of cortex and absent from the reticularis. Necrosis of adrenal cortical cells occurred in about one-half of the animals. The involved cells were scattered, less often grouped, in the outer one-third of the fascicularis. Rarely were they seen deeper in the cortex. Lipid depletion of moderate or marked degree was seen in all except 2 animals. This was studied in frozen sections stained with Sudan IV. In the majority, the depletion was usually greatest in, and often limited to, the outer half of fascicular and glomerular layers. Although measurements were not made, the cortex in a few adrenals was obviously reduced in thickness. The spleen of a number of rats showed marked hyperemia and lymphoid atrophy. The bone marrow studied in paraffin sections showed in many rats striking increase in erythropoietic activity and some decrease in number of granulocytes. The majority of the nucleated red cells were normoblasts. The only abnormal finding in the 13 control rats was lipid depletion of the adrenal glands of 4 rats; slight in 3, moderate in 1.

---

* The crystalline product employed had been isolated from a fermentation residue[7] and was furnished through the courtesy of Drs. E. L. R. Stokstad and B. L. Hutchings of Lederle Laboratories, Inc.

[7] Hutchings, B. L., Stokstad, E. L. R., Bohonos, N., and Slobodkin, N. H., *Science*, 1944, **99**, 371.

The adrenal hemorrhage and necrosis are almost completely prevented by thyroxin injections or by thyroid powder in the diet. Of 26 animals examined which had received thiourea, but no thyroxin or thyroid powder, 15 showed hemorrhage or necrosis or both of the adrenal. Of 15 animals which had received thyroxin injections in addition, and of 8 which had received thiourea and thyroid powder, 2 (one in each group) showed similar lesions. It would appear, therefore, that this damage to the adrenal glands is mediated in some way through the thyroid. The lipid depletion is not prevented by the injection of thyroxin or feeding thyroid powder.

Discussion. During the course of this work there appeared a report[8] describing the occurrence of granulocytopenia in rats fed a standard laboratory ration containing 0.5% of thiourea. The animals apparently developed neither leucopenia nor anemia but the average neutrophil percentage dropped from 32.7 to 5.4. It was reported that this granulocytopenia could be prevented by the inclusion of 5% of a crude liver fraction in the diet.

The anemia observed in our rats ingesting thiourea but receiving neither thyroxin nor thyroid powder apparently resulted in some way from the interference of the drug with thyroid function. This is suggested very strongly by the low incidence of anemia in the animals receiving thyroxin or thyroid powder in addition to the thiourea. These findings are in accord with the reported occurrence of anemia in clinical hypothyroidism and in thyroidectomized rats.[9]

It should be noted that the granulocytopenia in these experiments has been, comparatively, very difficult to correct. Rats with granulocytopenia induced by other methods have responded spectacularly and with considerable regularity to treatment for shorter periods and with smaller amounts of the vitamin. For example, in a recent study of the treatment of sulfasuxidine-induced granulocytopenia,[10] 11 of 14 animals responded to the administration of 25 μg of *L. casei* factor daily for 4 days. In these 11 animals the level of circulating granulocytes increased in 4 days from an initial average of 10 cells per cu mm to an average of 4530 cells per cu mm. Similar responses to identical therapy were obtained in granulocytopenic animals in which the dyscrasia was induced by diet alone,[11] by a deficiency of pantothenic acid,[6] or by a deficiency of riboflavin.[12] The granulocytopenia appearing in animals ingesting thiourea and receiving thyroxin or thyroid powder may differ therefore from the granulocytopenia resulting from these other conditions. It may be that the effect of the thyroxin and thyroid powder is to increase the animals' requirements for *L. casei* factor. A similar effect of thyroxin has been demonstrated for other vitamins of the B-complex.[13] The effect of the thiourea is conceivably due to a direct interference with some metabolic activity in the animal body.

It is of particular interest that the production of granulocytopenia by thiourea in these studies required the simultaneous administration of thyroxin or thyroid powder, each of which was given at a high level. These conditions are perhaps somewhat similar to those under which clinical agranulocytosis follows the use of thiourea.

Summary. Rats given thiourea in a purified diet develop anemia and, in lesser incidence, leucopenia. They also develop hemorrhage

---

[8] Goldsmith, E. D., Gordon, A. S., Finkelstein, G., and Charipper, H. A., *J. Am. Med. Assn.*, 1944, **125**, 847.

[9] Crafts, R. C., *Endocrinology*, 1941, **29**, 596.

[10] Endicott, K. M., Daft, F. S., and Ott, M., *Arch. Path.*, 1945, **40**, 364.

[11] Kornberg, A., Daft, F. S., and Sebrell, W. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 46.

[12] Kornberg, A., Daft, F. S., and Sebrell, W. H., *Arch. Biochem.*, 1945, **8**, 431.

[13] Drill, V. A., and Overman, R., *Am. J. Physiol.*, 1941-42, **135**, 474.

and necrosis of the adrenals. Animals which receive, concomitantly, thyroxin injections or thyroid powder become granulocytopenic and leucopenic, while the incidence of anemia and of adrenal hemorrhage and necrosis is greatly reduced. The granulocytopenia and leucopenia of these rats may be corrected by treatment with *L. casei* factor.

15258

Microbiological Determination of Amino Acids. IV. Lysine, Histidine, Arginine, and Valine

BEVERLY M. GUIRARD, ESMOND E. SNELL,* AND ROGER J. WILLIAMS.

From the Biochemical Institute and Department of Chemistry, The University of Texas, Austin.

Microbiological assays for amino acids have been reported in a series of papers from this laboratory.[1-3] A considerable portion of our more recent work has been anticipated by published results from other laboratories,[4-6] and we are therefore presenting only in abbreviated form additional methods for lysine, histidine, arginine, and valine and results of their application to 6 purified proteins: casein, gelatin, ovalbumin, β-lactoglobulin, horse hemoglobin, and silk fibroin.

Assay Procedures. Samples for assay were prepared by autoclaving (at 15 lb. steam pressure) 100 mg of the protein with 2 cc of 10% hydrochloric acid in a sealed tube for 24 hours. All assays were carried out as described by McMahan and Snell.[1]

Leuconostoc mesenteroides was employed for the lysine and histidine assays. The medium of McMahan and Snell[1] was modified for *L. mesenteroides* by increasing the phosphate content (Salts A) 8-fold and the arginine content 3-fold, and by altering the vitamin supplement (Solution 2) as described by Hac *et al.*[2] †

The lysine assay was incubated 63 to 65 hours at 32 to 34° C. The standard curve was constructed from response of the test organism to graded amounts of l (+)-lysine in the range 5-50 γ per 2.5 cc of medium. Growth response was measured turbidimetrically after dilution of the contents of each tube with 5 cc of a saturated aqueous solution of chlorothymol. Growth response may also be measured acidimetrically‡ using the micro quinhydrone-calomel-electrode technic or with the glass-electrode assembly described by McQuarrie and Jones.[7] When the assay tubes were to be titrated, 2% glucose was used instead of 1%, to permit formation of larger quantities of acid.

For the histidine assay, graded amounts of l(+)-histidine in the range 1-12 γ made up the standard curve. The culture tubes were incubated at 32 to 34° C for 72 hours. Shorter periods of incubation resulted in poor recoveries and drifting assay values.

---

* Present address: Department of Biochemistry, University of Wisconsin, Madison.

[1] McMahan, J. R., and Snell, E. E., *J. Biol. Chem.*, 1944, **152**, 83.

[2] Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1945, **159**, 273.

[3] Hac, L. R., and Snell, E. E., *J. Biol. Chem.*, 1945, **159**, 291.

[4] Dunn, M. S., Camien, M. N., Shankman, S., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1944, **156**, 715.

[5] Dunn, M. S., Camien, M. N., Shankman, S., and Rockland, L. B., *J. Biol. Chem.*, 1945, **159**, 653.

[6] Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, 1945, **160**, 35.

† The folic acid requirement is based on a concentrate with a potency of 40,000.

‡ The values obtained for the lysine content (uncorrected for moisture) of a casein hydrolyzate assayed turbidimetrically and acidimetrically were 7.0 and 7.1%, respectively.

[7] McQuarrie, E. B., and Konen, H. J., *Ind. Eng. Chem.*, Anal. Ed., 1944, **16**, 205.

TABLE I
Recovery of Amino Acids from Protein Hydrolyzates

Protein Hydrolyzate	Amino Acid	Amino Acid Content Found %	Amino Acid Added %	Total Content Found %	% Recovery
Casein	lysine*	7.3	7.0	14.3	100
’’	’’*	7.8	7.0	14.6	97.4
’’	’’*	7.34	7.0	13.9	94.0
’’	’’†	7.13	8.0	14.63	93
β-Lactoglobulin	’’†	9.87	9.83	19.71	100
Casein	histidine*	3.25	3.0	6.15	97
’’	’’*	3.11	3.0	6.17	101.9
Gliadin	’’*	1.99	2.0	3.89	95
Horse hemoglobin	’’*	7.63	7.6	15.3	99.1
’’ ’’	’’*	7.72	7.6	15.4	101
Casein	arginine†	3.74	4.0	7.94	105
β-Lactoglobulin	’’†	2.78	2.81	5.57	99.3
Casein	valine†	6.95	6.0	12.95	100
β-Lactoglobulin	’’†	5.5	5.8	11.0	94.6
Gelatin	’’†	2.4	2.4	4.85	102

Percentages are uncorrected for moisture.
* Determined with *L. mesenteroides.*
† Determined with *S. faecalis.*

TABLE II.
Lysine Content* of Several Proteins.

Sample	Lysine determined by *Streptococcus faecalis* %	Avg.	*Leuconostoc mesenteroides* %	Avg.	Other methods
Casein (Labco)	7.83, 7.39, 7.34, 7.76	7.6	7.50, 7.54, 8.0, 7.48, 7.70, 6.70	7.5	8.3[4] 7.7[6]
Gelatin (Knox)	5.2, 5.2	5.2	3.98, 3.75	3.87	5.8[6]
Ovalbumin†	6.04, 6.1, 5.85	6.0	5.6	5.6	6.6[6]
β-Lactoglobulin†	10.4, 10.0, 10.4, 10.4, 10.6	10.4	10.5	10.5	11.1[6]
Horse hemoglobin†	8.66	8.66	8.4	8.4	
Silk fibroin†	0.90, 0.90	0.90	0.58	0.58	0.60[4] 0.72[6]

* Percentages are expressed on the moisture-free basis.
† Kindly supplied by the late Dr. Max Bergmann.

The medium of McMahan and Snell[1] was modified for *Streptococcus faecalis* by doubling the phosphate concentration and by altering the vitamin supplement in the manner described by Hac *et al.*[2] The medium is very similar to that used by Stokes *et al.*[6] The assay was carried out on a 2.5 cc scale, incubated 16 hours at 30° C, and growth measured turbidimetrically after dilution of each culture with 5 cc of saturated aqueous chlorothymol. Stokes *et al.*[6] use 10 cc cultures, and follow growth titrimetrically after an incubation period of 40 hours. The determination of arginine, valine, and lysine was investigated using *S. faecalis* as test organism. Growth response increased with increasing concentration over the following ranges for the various amino acids: 8-50 γ *l*(+)-arginine monohydrochloride, 10-100 γ *dl*-valine, and 20-100 γ *l*(+)-lysine monohydrochloride per 2.5 cc of medium.

Results. Recoveries of amino acids added to protein hydrolyzates were good (Table I). There were no drifts in assay values as dosage levels of samples were increased; and assay values of protein samples tested at different times were reproducible (Tables II and III).

In Table II values for the lysine content of various proteins as determined with both *S. faecalis* and *L. mesenteroides* are given. Values for the histidine, arginine, and valine content of proteins are given in Table III. Values obtained by other investigators are

TABLE III.

Histidine, Arginine, and Valine Content* of Several Proteins as Determined Microbiologically.

Sample	Histidine†		Arginine		Valine		Values cited from the literature
	%	Avg.	%	Avg.	%	Avg.	
Casein (Labco)	3.3, 3.2, 3.2, 3.2, 3.2	3.2	3.82, 3.85, 3.53, 3.58, 3.60, 3.84	3.71	7.0, 6.5, 7.09, 7.09, 6.88	6.91	Histidine 3.1[5]; 2.8[6] Arginine 3.9[6] Valine 6.7[6]
Gelatin (Knox)	0.83, 0.83	0.83	8.73, 8.8	8.77	2.6, 2.6	2.6	Histidine 0.58[6] Arginine 9.1[6] Valine 2.7[6]
Ovalbumin	2.28, 2.4, 2.35, 2.45	2.4	5.72, 5.79, 5.83, 5.50	5.7	7.07, 7.66	7.36	Histidine 2.3[6] Arginine 5.9[6] Valine 7.0[6]
β-Lactoglobulin	1.72, 1.8, 1.67	1.73	2.98, 2.95, 2.70	2.88	6.07, 5.75	5.91	Histidine 1.5[6] Arginine 2.8[6] Valine 5.5[6]
Horse hemoglobin	8.2, 8.16, 8.13, 8.2, 8.06	8.2	2.8	2.8	9.0, 9.8	9.4	
Silk fibroin	0.41, 0.4	0.41	0.99	0.99	3.36, 3.46	3.41	Histidine 0.34[5]; 0.41[6] Arginine 1.11[6] Valine 3.5[6]

Histidine was determined with *L. mesenteroides*, arginine and valine were determined with *S. faecalis*.

* Values are expressed on the moisture-free basis.

† Employing the organism *L. delbruckii* 3 and a slightly different medium the values obtained for the histidine content of casein and gelatin—3.2 and 0.84, respectively—are in excellent agreement with those above.

also given for comparison.

Discussion. Values for the lysine content of proteins obtained with *S. faecalis* and *L. mesenteroides* are in good agreement with each other and with values cited from the literature, except in the cases of gelatin and silk fibroin. The value obtained with *L. mesenteroides* for gelatin appears to be low, and that for silk fibroin with *S. faecalis* appears to be high when compared with the data cited. The value obtained for the lysine content of horse hemoglobin is in excellent agreement with that obtained by the isotope dilution method.[8]

The single value (2.8%) for the arginine content of horse hemoglobin probably should be disregarded since it is much lower than the values 3.4, reported by McMahan and Snell,[1] and 3.7, reported by Foster.[8] The value (9.4%) for the valine content of horse hemoglobin is somewhat high as compared with the value, 8.8%, reported by McMahan and Snell.[1] Recent values for the histidine content of horse hemoglobin are unavailable. The agreement among other values reported in Table III is excellent.

Summary. A method of assay for histidine and lysine employing *Leuconostoc mesenteroides* as test organism has been developed. This method is compared with those of Dunn *et al.* [4,5] A method of assay similar to that of Stokes *et al.*[6] for lysine, arginine, and valine using *Streptococcus faecalis* as test organism has been independently developed and studied.

With few exceptions, the data reported agree closely with those obtained by previous investigators with microbiological or isotope dilution methods.

---

[8] Foster, G. L., *J. Biol. Chem.*, 1945, **159**, 431.

15259

The Nature of Circulating Estrogen.*

Clara M. Szego, and Sidney Roberts. (Introduced by William Malamud.)
From the Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

In the course of investigations on the physico-chemical nature of circulating estrogen, we have found a large and constant portion of the latter to be closely associated with the blood proteins. Coincidentally, we have observed that blood estrogens will dialyze quantitatively past a collodion membrane.

Methods. The methods used for the extraction of blood estrogens will be published in detail elsewhere. In brief, the procedure generally consisted of precipitation of the blood proteins with acetone. The supernatant fraction was combined with the acetone-ether washings of the precipitate, after which the two fractions were treated separately. In all instances, precipitation and washing of the protein was done in the cold.

After evaporation of acetone and ether from the supernatant fraction, the latter was hydrolyzed with 4% H_2SO_4. The freed estrogen was then extracted with ether, and the water-washed extract was made up in a small amount of olive oil for subsequent assay.

The previously undenatured protein precipitate was subjected to partial alkaline hydrolysis with 0.1 N NaOH. After neutralization with CO_2, this material was extracted with ether and made up in olive oil.

Other methods of estrogen extraction included alcohol-ether precipitation, and ammonium sulfate precipitation followed by prolonged hot alcohol-ether Soxhlet extraction of the protein.

Portions of the same or similar bloods, as well as pure solutions of various natural estrogens, were also subjected to dialysis against distilled water through sausage casing (Visking, 25/32″ diam.) The dialysate

---

* Aided by grants from the Foundation for Applied Research and G. D. Searle & Co.

TABLE I.
Distribution of Blood Estrogen.

Sample	Method	Volume cc	Fraction	Treatment of protein before ether extraction	Estrogen as μg α-estradiol per 100 cc		% estrogen in protein fraction
						Total	
Rabbit* Serum	Acetone Pptn.	72	Supernatant	-	<0.34		
			Protein	Alkaline partial hydrolysis	0.47	0.81	>58
Cow* Whole blood†	” ”	100	Supernatant	—	0.10		
			Protein	” ”	0.20	0.30	67
Pregnant cow (6 mo.) Whole blood†	” ”	100	Supernatant	—	0.15		
			Protein	(1) Aqueous extr.	0		
				(2) Alk. partial hydr.	0.23	0.38	61
				(3) Hydr. at pH 1	0		
Pregnant cow (6 mo.) Whole blood†	Alcohol-ether Pptn.	65	Supernatant	—	0.12		
			Protein	Alk. partial hydr.	0.24	0.36	67
Pregnant human (7 mo.) Whole blood†	$(NH_4)_2SO_4$ Pptn.	100	Supernatant	—	0.20		
			Protein	Soxhlet‡	0.40	0.60	67

* Gonadotrophin treated.

† Preserved with potassium oxalate 0.2%.

‡ Continuous extraction with hot alcohol-ether for 48 hours.

TABLE II.
Dialysis of Pure Estrogens

Estrogen	μg inside before dialysis	Form	Hrs. of dialysis	μg found in	μg found out	% recovery	% dialysis*
α-Estradiol	10	Na Salt	24	4.0	6.4	104	74
"	10	"	48	1.0	8.0	90	94
"	1.0	"	48	—	0.9	—	105
"	9.5	Free	48	4.5	4.0	90	35
"	2.0	"	48	—	0.4	—	25
β-Estradiol	10	Na Salt	48	—	8.3	—	100
Estrone	2.0	"	48	—	1.8	—	106
Estriol	30	"	48	—	24.8	—	98

* Based on theoretical considerations with respect to volume ratios across the collodion membrane.

was ether-extracted before and after acid hydrolysis and prepared for injection in olive oil. The material inside the cellophane bag was often analyzed for estrogen coincidentally; in the case of blood, the acetone procedure outlined above was employed. In all instances, dialysis was carried out in the cold (2°C) in a mechanical shaking device.

Biological assay for estrogenic activity was by a modification[1] of the 6-hour uterine-weight method of Astwood.[2] The index employed was the increase in the ratio of uterine weight to body weight in immature female rats. Most of the results were represented arbitrarily in terms of alpha-estradiol equivalents.

Results. Blood and sera obtained from normal and pregnant cows, normal and gonadotrophin-injected rabbits, and pregnant women were analyzed for estrogen.

It was uniformly found that only one-third of the total estrogen was present in the acetone supernatant fraction from which the protein had been removed without denaturation (Table I). This portion was apparently almost entirely present in a conjugated form (probably as an ester), since it could be extracted with ether only after acid hydrolysis. The remainder, or two-thirds, existed associated with the protein in a form liberated by weak alkaline hydrolysis. Subsequent hydrolysis at pH 1 did not increase the yield. It may be noted that only very small amounts of estrogen were present in all instances.

In the course of related studies it was found that pure estrogens will dialyze quantitatively past a collodion membrane. This is illustrated in Table II. Among the estrogens studied, free estradiol (which we have not found to any significant degree in blood) dialyzes too slowly to reach equilibrium in a reasonable length of time. This may be due to its hydrophobic nature in a salt-free environment. Otherwise, dialysis appeared to reach theoretical completion in about 48 hours. Dialysis was not complete in 24 to 26 hours. This is illustrated by the incomplete dialysis of the sodium salt of alpha-estradiol after 24 hours (Table II) and of pregnant cow plasma and laked cells after 26 hours (Table III). Similar samples had dialyzed completely after 36 to 48 hours.

Table III shows that the amount of estrogen obtained from a 36 to 72 hour dialysate of blood was approximately equivalent to that found by exhaustive chemical extraction procedures (Table I). The optimal volume ratio of material inside the collodion bag to that outside was found to be about 1:5. Dialysis was incomplete where this condition was not met, as in the case of the whole blood sample from the gonadotrophin-injected cow (Table III). In this case, the volume ratio was only 1:2.

Discussion. Previous studies on natural blood steroids having hormonal activity have almost uniformly neglected the protein fraction, in spite of the fact that a large amount of cholesterol is found closely associated with the latter.[3] However, a variable amount of estrogen has been found associated with

---

[1] Pincus, G., and Werthessen, N., personal communication.

[2] Astwood, E. B., *Endocrinology*, 1938, **23**, 25.

[3] Tronsegaard, N., and Koudahl, B., *Z. Physiol. Chem.*, 1926, **153**, 111.

TABLE III.
Dialysis of Estrogen in Blood.

Sample	Volume cc	Duration of dialysis in hrs.	Estrogen as μg α-Estradiol per 100 cc
Cow serum	160	48	0.25
Cow*			
Whole blood†	200	46	0.20
Plasma†	100	46	0.40
Laked cells†	100	46	0.35
Pregnant cow (6 mo.)			
Whole blood†	50	36	0.34
Plasma†	100	26	0.10
Laked cells†	100	26	0.20
Pregnant human			
Whole blood† (3 mo.)	100	72	0.22
Whole blood† (5½ mo.)	100	72	0.55

* Gonadotropin treated.
† Preserved with potassium oxalate 0.2%.

the protein in mare serum[4] and in human pregnancy serum.[5] This bound estrogen could be liberated by prolonged strong acid hydrolysis, but not by refluxing 4 hours with alcohol, nor by ultrafiltration or tryptic digestion.

Our data suggest that an equilibrium exists in blood between estrogen and protein. Thus, two-thirds of the total estrogen is normally closely associated with the protein. The remainder is apparently present in an esterified form in the aqueous phase. The hydrophilic nature of this esterified estrogen may be involved in the formation of the protein complex. It is improbable that the latter represents merely simple adsorption, since the relative amount of estrogen found associated with the protein by different extraction procedures is quite constant (Table I).

The estrogen-protein complex is of such a nature that dialysis results in its progressive dissociation. It is probable that this is accomplished by preserving the ratio between dissociated and protein-bound estrogen inside the collodion bag. As dialysis proceeds, gradually removing the protein-free estrogen, further dissociation of the estrogen-protein complex is permitted. This postulated equilibrium, then, seems to obey the law of mass action, enabling dialysis to proceed toward completion, depending upon the concentration gradient of estrogen across the membrane.

The above results bear on the fundamental problem of steroid transport and activity *in vivo*, and suggest that estrogen bound to protein is potentially available for physiological action by dissociation at the cell membrane.

Summary. The total estrogen content of blood and sera obtained from normal, pregnant and gonadotrophin-injected animals and women was uniformly low. Values approximating 0.5 μg of alpha-estradiol equivalents per 100 cc of blood were found.

Two-thirds of the total estrogen was closely associated with the protein fraction. The remaining one-third existed mainly in a hydrophilic, non-protein conjugated form.

Blood estrogens, as well as pure solutions of the sodium salts of estrone, alpha- and beta-estradiol, and estriol, were shown to dialyze quantitatively past a collodion membrane.

The authors wish to express their indebtedness to Dr. Samuel C. Gwynne and to Mr. Raymond Umbaugh for furnishing them with some of the blood samples.

---

[4] Haussler, E. P., *Festschrift E. C. Barrell*, 1936, 327 (Basel).

[5] Rakoff, A. E., Paschkis, K. E., and Cantarow, A., *Am. J. Obstet. and Gynec.*, 1943, **46**, 856.

15260 P

Effect of Certain Amino Acids on Healing of Experimental Wounds of the Cornea.

ALEXANDER J. SCHAEFFER. (Introduced by H. J. Deuel, Jr.)
From the Department of Biochemistry, University of Southern California, Los Angeles, Calif.

As a non-vascularized tissue, the cornea is unique from a biological point of view. It receives its nourishment by diffusion of fluids through the entire corneal substance. In trauma this diffusion may be disturbed leading to vascularization and loss of transparency.

Earlier many attempts were made to nourish the elements of tissue cultures and to increase their rate of proliferation *in vitro*.[1] The attempt to influence the regeneration of corneal defects by embryonic tissue extracts encountered many difficulties. Fischer[2] found that in tissue cultures *in vitro* the embryonic extracts could be replaced by an artificial medium in which amino acids furnished the building stones for the synthesis of protoplasm. Cystine, which seemed to function both as an energy-furnishing ingredient and as a growth catalyst, apparently played a major role in this medium. This special function of cystine is probably connected with its sulfur content, as free sulfhydryl groups often activate proteolytic systems in the process of growth.

Encouraged by Fischer's results the writer has made an attempt to influence the regeneration of experimental wounds of the cornea by administration of amino acids to this tissue *in vivo*. For the first experiment 36 guinea pigs were used. The technic employed was a modification of the method of Gundesen and Liebman.[3] Superficial vertical incisions were made across the cornea into the epithelium of both eyes by means of a Castroviejo double knife with blades 2 mm apart. The cornea was immediately stained with fluorescein and the epithelium between the two lines evenly abraded with a spatula. The corneae were periodically stained and the fluorescein-positive areas registered on a chart drawn on logarithm paper. The chart consisted of a ring symbolizing the limbus with a vertical double line in the center. By counting the number of stained and unstained squares of the paper, a quantitative expression of the healing process could be obtained.

In 18 of these animals (1,a) the amino acid solution was dropped into the right eye every hour while the left control eye received a corresponding amount of physiological saline. In the remaining 18 animals (1,b) an ointment containing the amino acids was applied twice a day to the right eye together with a 5% boric acid ophthalmic ointment which was applied to both eyes.

The same experiment was repeated on 6 other animals (2) but instead of a stripe, a round erosion, 3.5 mm in diameter, was made with a trephine in a similar manner. The amino acid solution was made in 6% NaCl and contained 2 mg cystine, 5 mg proline, 6 mg asparagine and 14 mg glutamine per ml, and was adjusted to pH 7.2. The ointment was prepared by emulsifying 70 g aquafor with 30 ml amino acid solution which contained the aforementioned amino acids in 10-fold concentration.*

A complete regeneration of the corneal defect in the eyes treated with amino acids was achieved within 12 to 42 hours (in less than 30 hours in an average), while the healing process in the control eyes required 55 to 120 hours.

In a third series of experiments the effect of amino acids on healing of deep non-per-

---

[1] Bisceglie, V., and Schaeffer, A. J., *Die Gewebezuchtüngen in vitro.*, Springer, Berlin, 1928.

[2] Fischer, A., *Acta Physiol. Scandinav.*, 1940, **2**, 143.

[3] Gundesen, T., and Liebman, S. D., *Arch. Ophth.*, 1944, **31**, 29.

* I am indebted to Dr. Ernest Geiger for suggestions and for the material used in these experiments.

TABLE I.

Summary Table Showing Type of Corneal Injury and the Average Healing Time in Control and Amino Acid-treated Animals.

Expt. No.	Experimental injury	Treatment		No. of Animals	Healing time*	
		Control	Amino acid treated		Control eye	Treated eye
Guinea pigs						
1,a	Vertical 2 mm. erosion	Physiol. saline hourly	Amino acid soln. hourly	18	64(55-108)	22(15-32)
1,b		5% boric acid ointment twice daily	5% boric acid ointment containing amino acids twice daily	18	78(66-120)	26(12-42)
2	Round erosion, 3.5 mm	physiol. saline hourly	Amino acid soln. hourly	6	62(50-112)	21(14-38)
Rabbits						
3,a	Deep non-perforating trephine wound	” ”	” ” ”	2	95(86-104)	35(28-42)
3,b		5% boric acid ointment twice daily	5% boric acid ointment containing amino acids twice daily	10	108(96-120)	40(32-56)

* The first figure gives the average and the values in parentheses the minimum and maximum values of the individual experiments.

forating trephine wounds of the cornea was examined. In 12 rabbits a central defect 2.5 mm in diameter and 0.4 mm deep was made into the substance of the cornea of both eyes. The right eye was treated in 2 animals (3,a) with hourly administration of drops containing amino acids while the left eye received saline solution. The other animals received ointment applications as in the first experiment (3,b).

In the eyes treated with amino acids, the corneal defect was covered with epithelium within 32 to 56 hours and in the control eyes between the 4th and 5th day. The biomicroscopic parallel with the histological examination showed that in the amino acid-treated eyes the same phase of the healing process is reached after 2 days which the control eyes reached only after the 6th day of experiment.

The results of treatment of over 100 clinical cases in man with the same ointment will be published elsewhere. Although it is difficult to evaluate these data quantitatively since the ointment was never applied alone, it would appear that the application of amino acids accelerates the healing of corneal wounds particularly when ulcerative processes are involved.

15261

Accidental Air Embolism and Fibrin Formation in the Heart of Rabbit.

JOHN AUER, AND HUGO KRUEGER.

From the Department of Pharmacology, St. Louis University School of Medicine, St. Louis, Mo.

During operations on the spinal column, cord and skull, a number of rabbits died more or less acutely and their death was at first attributed to that diffuse symptom-complex "vascular shock." Autopsies, however, soon revealed cleaner factors that played an undoubted rôle in the fatal outcome; gas bubbles were seen in the pulmonary artery associated at times with fibrin formation in the heart, especially in the right ventricle.

The danger of air embolism during operations on the neck and chest, because of negative intra-thoracic pressure, is well known[1] though possibly not fully appreciated and we shall show that this danger also exists for operations on the skull. The experimental and clinical literature contains numerous reports of air embolism following the injection of air or oxygen into various cavities and spaces of the body.[2] It will be further shown that fibrinous masses may develop acutely in the heart during air embolism; this observation has only been reported once before as far as we know: Richardson, Coles and Hall[3] observed occasional flecks of fibrinous material around the chordae tendineae of the right ventricle, and in 3 cases saw a friable clot of blood and air that possibly acted as a ball valve against the pulmonary artery; these investigators used nembutalized dogs and injected air into a femoral vein.

Although Kleinschmidt[4] has published an excellent experimental study of air embolism in rabbits, we shall report our own findings because Kleinschmidt and other experimenters injected air intravenously, while our work deals with air embolism following operations on the skull and spinal region.

Technic. The rabbits were generally narcotized by the subcutaneous injection of 150 mg of sodium barbital per kilo and operated 30 to 45 minutes later; in a few instances morphine or ether was used. The chief operations were tracheotomy; exposure of the thoracic spinal column and cord; transection of the cord combined at times with pithing below the site of transection; exposure of the diploic veins in the parietal bones or removal of the outer lamella of the squamous portion of the occipital bone. In some series, the vagi were sectioned in the neck; in others they were left intact. Inspiratory dyspnoea was often produced for short periods by an ordinary flap-valve slipped over the tracheal cannula. In the curare experiments, artificial respiration was given at the rate of 30 per minute. The blood pressure was recorded by a mercury manometer in a number of experiments.

If the animal succumbed acutely, an autopsy was carried out at once; otherwise a biopsy was performed 30 to 55 minutes after the operative interference.

Experimental Results. Cord operations were carried out on 28 rabbits. In 22 both vagi were cut and 11 died acutely; 8 of these 11 rabbits revealed air bubbles in the pulmonary artery; the remaining 11 rabbits were biopsied and one showed both air bubbles in the pulmonary artery and a good amount of fibrin in the right ventricle; a second rabbit revealed no air bubbles but both ventricles showed some fibrin shreds. In 6 rabbits, the vagi were left intact; none of these died acutely but a biopsy revealed air in the pulmonary artery of 2. Thus 11 out of 28 rabbits showed air bubbles in the pulmonary artery.

Skull operations were performed on 9 rabbits and the vagi were cut in all. Acute death occurred in 4 and 3 of these showed air bubbles in the pulmonary artery. Biopsy was necessary in 5 and 2 of these subacute cases showed air bubbles in the pulmonary artery. In one interesting experiment, both vagi cut, air bubbles were seen coursing through the exposed diploic veins of a parietal bone during deep inspiration. Biopsy was performed 27 minutes after the air was seen entering the diploic veins. At that time, the blood pressure was 80 mm Hg and the respiration 172 per minute. On opening the chest in the midline, the heart was beating powerfully and regularly, the pulmonary artery was distended by air bubbles and the right side of the heart was fuller than the left. Section of the heart revealed no air bubbles in the right atrium and ventricle, but the right atrium contained a small amount of fibrin while all other chambers were free of fibrin. It seems probable that this rabbit would have recovered.

---

[1] Ceelen, W., in *Henke-Lubarsch Handbuch der speziellen pathologischen Anatomie und Histologie*, Berlin, Julius Springer, 1931, III, Part 3, 119.

[2] Reviews of experimental and particularly clinical cases: Breyfogle, H. S., *J. A. M. A.*, 1945, **129**, 342; Wolffe, J. B., and Robertson, H. F., *Ann. Int. Med.*, 1935, **9**, 162.

[3] Richardson, H. F., Coles, B. S., and Hall, G. E., *Canadian Med. Assn.*, 1937, **36**, 584.

[4] Kleinschmidt, O., *Arch. f. Klin. Chirurgie*, 1915, **106**, 782.

In this series, 5 out of 9 rabbits showed air bubbles in the pulmonary artery.

Cord operations under curare and artificial respiration were studied in 16 barbitalized rabbits; the vagi were cut in 6. In this series, only one rabbit out of 16 showed air bubbles in the pulmonary artery and this exception was probably caused by imperfect curarization. No fibrin was found in any heart.

Fibrin Networks in the Heart. In a number of rabbits dying acutely or biopsied 30 minutes more or less after the operative procedure, a fibrin network was noted in the right ventricle, though it also occurred in the right atrium and sometimes in the left ventricle. The amount of fibrin varied considerably. In one extreme instance the elastic, reddish or reddish-black network interlaced the papillary muscles and chordae tendineae of the right ventricle practically filling this cavity and a portion extended up to the pulmonary artery; in most instances, however, the amount was much less. When fibrin occurred in the left ventricle, it was present only as a thin film.

In the series of 16 acutely fatal cases, 10 revealed fibrin in the heart; in 2 or 3 of these 10 fibrin-containing hearts, no air bubbles were detected, but air bubbles were seen in the other 7 hearts. The vagi had been cut in all 16.

In 21 subacute experiments, fibrin was found in the heart of 7 cases and in 4 of these no air was seen in the pulmonary artery; the remaining 3 showed air in the pulmonary artery. In 3 instances, fibrin was found in both ventricles without any sign of air. One rabbit (vagi intact) showed air bubbles but no fibrin; the remaining 13 rabbits (vagi cut in 9, intact in 4) showed neither air nor fibrin in the heart.

An instructive accident demonstrated that air embolism could produce the formation of fibrin in the left ventricle only. This happened when the lung alveoli were ruptured during artificial respiration. Immediate autopsy showed air bubbles in the aorta, carotid and mesenteric arteries, but no air was seen in the right side of the heart and pulmonary artery. In this heart, the left ventricle showed fibrin, while the right ventricle contained none.

Since the right ventricle occasionally contained definite fibrin networks, it might be assumed that particles could be dislodged and finally plug some of the small subdivisions of the pulmonary artery. No definite signs of embolism, however, were detected in the lungs of rabbits that died acutely or that were biopsied although these animals showed fibrin in the right ventricle. Very frequently section of the various lobes of the lung showed reddish to red-black polygonal areas that were a few millimeters in diameter; these congested areas were surrounded by larger, grayish-white areas that were full of air and were largely bloodless. Microscopic sections also gave no evidence of embolism. The time, however, between the formation of fibrin and acute death or biopsy was probably too short to permit adequate stasis for infarction to occur.

Pulmonary edema in the great majority of our experiments was slight and limited at times to one lobe. This is contrary to Kleinschmidt's results ([4] p. 798) who always found strong pulmonary edema; Richardson, Cole and Hall also report marked pulmonary edema ([3] p. 587).

Discussion. The recorded experimental results show unmistakably that air embolism during operations on the spinal column, cord and skull in heavily barbitalized rabbits is an ever-present danger and that this air embolism may cause acute death or a subacute state from which recovery probably could ensue. Thus in 37 spinal and skull operations, 16 rabbits revealed air bubbles in the pulmonary artery. It is therefore clear that the diagnosis "shock" as the cause of death in rabbits after operations on the skull, neck and chest should not be made until a careful autopsy has determined whether or not air embolism was the primary factor. This diagnosis, however, is rendered more difficult by the fact that the air bubbles may have been absorbed before the autopsy was made. Here a practically unknown sign is at times available that indicates the previous existence of air emboli and that is the presence of fibrin networks, particularly in the right ventricle. In our series, 7 rabbits showed fibrin networks in the heart without any detectable air bubbles.

It seems obvious that this fibrin must have been formed by churning of the air-blood mixture in the right ventricle during cardiac activity. Why every heart with air in the pulmonary artery did not show fibrin is probably due to a number of factors. If the air bubbles are promptly expelled into the pulmonary artery, no defibrination of the right ventricular blood can occur, and air in the pulmonary artery does not measurably obstruct the flow of blood according to Kleinschmidt ([4] p. 802). The longer the right ventricle is able to churn the blood-air mixture, the greater the chance of causing defibrination. If the amount of air is too large, right ventricular failure ensues, as is demonstrated by the bulging dark-colored right atrium and ventricle and the contracted, pink, and empty left atrium and ventricle. Posture of the body also is important; Kleinschmidt ([4] p. 791) states that air is expelled with greater difficulty when the apex of the heart is at a higher level than the base. Another factor is the coagulability of the blood; we attempted to correlate the coagulation times before and after the experiment with the appearance of fibrin in the heart, but our results in 10 experiments permitted no definite conclusion.

Our data do not permit a definite statement about the rôle played by section of the vagi in the production of air embolism because the number of controls with vagi intact is inadequate; it seems, however, that section of the vagi is not a vital factor. Acute death, however, was more frequent in the cord series of 28 rabbits when the vagi had been cut.

Summary. 1. In barbitalized rabbits subjected to operations on the spinal column, cord and skull, air embolism is not a negligible danger; 14 out of 37 rabbits showed air bubbles in the pulmonary artery. Acute death after such operations in the rabbit must not be diagnosed as "vascular shock," tacitly implying a nervous component, until a careful autopsy has excluded air embolism.

2. Air bubbles were observed entering and coursing through exposed sections of the diploic veins of the parietal bone during inspiration.

3. Absence of air bubbles in the right heart and pulmonary artery does not exclude air embolism for the air may have been absorbed. The diagnosis of air embolism may still be made if fibrin is found in the heart. Fibrin formation in the heart, especially the right ventricle, was seen 6, possibly 7, times when no air bubbles were detectable anywhere. Occasionally a slight amount of fibrin may also be detected in the left ventricle. Here apparently some air bubbles passed through the pulmonary circuit and entered the left ventricle. Fibrin may occur only in the left ventricle; this was seen once when artificial respiration ruptured lung aveoli and air entered the left heart and arterial system only.

4. No definite evidence of pulmonary infarction was seen in the lungs on macroscopic and microscopic examination. It seems probable that pulmonary infarction would have occurred had some of the animals been allowed to survive for longer periods of time.

5. Pulmonary edema was slight in the majority of our experiments.

15262

Experimental Evidence on the Cerebral Origin of Muscle Spasticity in Acute Poliomyelitis.*

David Bodian. (Introduced by H. A. Howe.)

From the Poliomyelitis Research Center, Department of Epidemiology, Johns Hopkins University, Baltimore, Md.

One of the common symptoms of acute poliomyelitis in man and in experimental primates is the muscular rigidity, associated with hyper-reflexia, which often precedes flaccid paralysis. The considerable recent interest in this phenomenon has been associated with conflicting views as to its pathogenesis, its possible role in producing chronic deformities, and its appropriate treatment. Recent observations specifically directed at determining the characteristics and distribution of muscle spasticity in acute human poliomyelitis are in agreement with respect to the following points: 1. Muscle spasticity in acute poliomyelitis is a reflex phenomenon associated with increased stretch reflexes.[1-8] 2. Spasticity in acute poliomyelitis has a widespread occurrence in the skeletal musculature, may be present in both flexors and extensors, and may occur in partly weakened muscles as well as in muscles of normal strength.[1,2,4,6] Our observations in the rhesus monkey are in agreement with these findings in human subjects, although in some monkeys spasticity is not observed. This may result because of absence of the causes of this symptom, because of failure of detection, or because severe motoneuron paralysis occurs so quickly as to shorten greatly the period of spasticity or to preclude its occurrence. In some monkeys preparalytic spasticity may be so severe that awkwardness of gait and posture is apparent, and the resistance of extremities to passive movement is claspknife in character. At this stage a fine tremor may be present.

The fact that the lesions of paralytic poliomyelitis occur in a number of nervous centers concerned with muscle function in the brain as well as in the spinal cord, makes possible a variety of hypotheses regarding the pathological site of origin of this symptom. Added to the difficulty of analysis imposed by the widespread nature of lesions usually present in fatal human cases, is the fact that the symptom in question has usually disappeared by the time death occurs. In spite of these difficulties, it is most commonly assumed that the lesions which are responsible for the symptom of spasticity are those in the region of the spinal cord which supplies the local reflex mechanism.[3,6,8,9] Others attribute the hyperirritability of the stretch reflex to possible irritation of the muscle, nerve, posterior roots, or meninges by the virus,[4] or to lesions of sensory neurons in the posterior horns as well as in sensory ganglia.[7] None of our observations on pathological changes in many human or monkey specimens has suggested to us that lesions in these locations are quantitatively sufficient to be of primary importance in producing

---

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

[1] Schwartz, R. P., and Bouman, H. D., *J. A. M. A.*, 1942, **119**, 923.

[2] Bouman, H. D., and Schwartz, R. P., *N. Y. State J. Med.*, 1944, **44**, 147.

[3] Schwartz, R. P., Bouman, H. D., and Smith, W. K., *J. A. M. A.*, 1944, **126**, 695.

[4] Watkins, A. L., Brazier, M. A. B., and Schwab, R. S., *J. A. M. A.*, 1943, **123**, 188.

[5] Brazier, M. A. B., Watkins, A. L., and Schwab, R. S., *New England J. Med.*, 1944, **230**, 185.

[6] Moldaver, J., *J. A. M. A.*, 1943, **123**, 74.

[7] Moldaver, J., *J. Bone and Joint Surg.*, 1944, **26**, 103.

[8] Kabat, H., and Knapp, M. E., *J. A. M. A.*, 1943, **122**, 989.

[9] Kabat, H., and Knapp, M. E., *J. Pediat.*, 1944, **24**, 123.

the generalized spasticity so common in acute poliomyelitis. It is finally obvious that the presence of lesions in the motor cortex and in brain stem centers which function to modify muscle activity, has occasionally suggested that these lesions are sources of interference with inhibitory motor mechanisms.

The difficulties of analysis of the pathogenesis of spasticity in humans can be partly overcome in rhesus monkeys because of a fortunate circumstance. We have shown that in rhesus monkeys inoculated intranasally or intracerebrally, the progress down the brain stem of lesions produced by virus activity may occasionally be arrested spontaneously at any point before reaching the spinal cord.[10,11] Such non-paralytic attacks of poliomyelitis in rhesus monkeys are not accompanied, in our experience, by severe spastic phenomena, but as a rule the lesions in the brains of such animals are not severe. However, brain lesions *may* be very severe in some inoculated animals in the preparalytic period, when spasticity is the predominant neurological symptom. It was thought possible that in some animals in this stage, virus activity or lesions might not have progressed as far as the spinal cord. With severe infections induced by most of the virus samples we have used, this is rarely true, since lesions are usually found to be present in both the lumbar and the cervical cord by the time that spasticity is well-marked.[†] With the Lansing strain,[12] however, we have found that not only do the arms usually show paralysis first but, in addition, the onset of paralysis in the legs is often somewhat delayed as compared with other strains.[‡] Consequently, an attempt was made to kill certain animals inoculated intracerebrally with Lansing virus in the preparalytic period after definite spasticity had become manifest. It was found in 2 cases that marked spasticity and hyperreflexia were present in arms and legs *before* lesions or virus (in one case) could be demonstrated in the lumbo-sacral cord, although lesions and virus activity were present in the brain and cervical cord. An additional animal which had been inoculated with the Rockefeller MV strain in 1939 and had been killed in the preparalytic period for another purpose was subsequently found to show a similar pathological picture. It was thus apparent that lesions in the brain alone could produce the spasticity of acute poliomyelitis.

The protocols of these animals are of sufficient importance to be presented here:

Rhesus B474. This was an adolescent rhesus monkey in good condition.

Nov. 7, 1945. Under nembutal anaesthesia a trephine hole was prepared over the central sulcus in the midline, and 0.4 cc of a 10% rhesus spinal cord suspension containing the Lansing strain of virus was inoculated into each dorsal thalamus.

Nov. 12. No abnormal signs were noted.

Nov. 13. The animal was observed to be very awkward and stiff in climbing and running. Extensors and flexors of arms and legs were tense and abnormally resistant to passive movement. Deep reflexes of arms and legs were hyperactive. Head tremor was present. No weakness or difference in strength between opposite limbs could be detected by careful examination.

---

[10] Bodian, D., and Howe, H. A., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 135.

[11] Bodian, D., and Howe, H. A., *Bull. Johns Hopkins Hosp.*, 1945, **76**, 1.

[†] Six rhesus monkeys killed while showing generalized spasticity in the preparalytic period after inoculation with one of 3 virus strains recently isolated from humans (Sudeck, 1941; Riley, 1943; Frederick, 1945) were found to have poliomyelitic lesions in both cervical and lumbar cord segments.

[12] Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 2302.

[‡] This curious strain characteristic can be illustrated by the fact that 61 of the last 81 rhesus monkeys inoculated intrathalamically with Lansing virus in this laboratory were first paralyzed in one or both arms, whereas only 6 were first paralyzed in one or both legs, and the remaining 14 exhibited both arm and leg weakness on the first day of paralysis. In contrast, only 17 of 82 monkeys inoculated similarly with one of 6 other strains isolated in this laboratory were first paralyzed in one or both arms, 20 were first paralyzed in one or both legs, and 45 had both arm and leg weakness on the first day of paralysis.

TABLE I.
Comparison of Numbers of Normal and Pathological Motoneurons in Cervical Enlargement of Rhesus B474, Based on Counts of Lateral Columns of 45 Equally Spaced 15 μ Sections (Preparalytic Poliomyelitis).

	Left	Right	Expected on basis of studies of normal controls
Normal	400	314	450-500
Chromatolytic	44	93	—
Necrotic	9	16	—
Estimated deficit§	0-47	27-77	—
Total	453-500	450-500	450-500

§ Motoneurons absent because of complete autolysis or phagocytosis.

Under ether anaesthesia, the animal was perfused through the aorta with 10% formalin containing 1% acetic acid. The brain and spinal cord were removed for histological study. Serial sections at 15 μ were prepared of the cervical cord from C6 to T1 inclusive, and of the lumbosacral cord from L2 to S1 inclusive. All sections of the above 8 lumbosacral segments were stained with gallocyanin and examined.

Histopathological Findings. Sections of the cervical cord contained typical early lesions of poliomyelitis, with moderate perivascular and diffuse leucocytic infiltration, and neuronal changes. Relatively few neurons were destroyed, but a considerable number showed various degrees of chromatolysis. The left anterior horn was almost free of lesions except in the lower part of C8 and the upper part of T1. Actual motoneuron destruction on the right side was limited to segment C7, although various degrees of chromatolysis were present in all cervical segments. An actual count of the anterior horn cells in 45 sections taken at intervals through the cervical enlargement showed the distribution of normal and affected motoneurons seen in Table I.

A quantitative study of the relationship of motoneuron destruction to muscle weakness, now in progress, indicates that the amount of motoneuron damage in the cervical cord was far below the level which produces clinically evident weakness.

All sections of the lumbosacral enlargement (L2 to L7, and S1) were examined. No lesions of poliomyelitis were detected.

The brain was imbedded in celloidin and sectioned at 60 μ. Every 20th section was stained with gallocyanin and examined. A typical distribution of severe focal and diffuse infiltrative lesions and of neuronal destruction was found. Severest lesions were observed in the motor cortex (areas 4 and 6 of Brodmann), dorsal thalamus, fields of Forel, substantia nigra, superior colliculi, midbrain tegmentum, reticular formation of hindbrain, vestibular nuclei, and basal cerebellar nuclei. Neuron destruction, however, was most marked in the reticular formation and in the large cells of the vestibular nuclei.

Rhesus 959. This was an adolescent female, slender but active.

March 17, 1939. Under ether anaesthesia a trephine hole was prepared over the right motor cortex and 0.2 cc of a 20% rhesus cord suspension containing the Rockefeller Institute MV strain of virus was injected in the cortex of the leg region.

March 23. Rectal temperature had risen from a base-line of 102°F. on March 21 to 105.2°F. No muscular weakness was detected by observation of movements or by palpation. The right leg, however, was observed to be definitely more spastic than the left leg or arms, and the right knee jerk was more active than the left. Under ether anaesthesia, the animal was perfused through the aorta with 10% formalin containing 1% acetic acid. The brain and spinal cord were removed for histological study. Serial celloidin sections at 30 and 60 μ were prepared of the brain, and paraffin sections at 15 μ of the cervical and lumbar cord, and these were stained with gallocyanin.

Histopathological Findings. Typical inflammatory lesions were found at all levels of the cervical cord examined from C6 to T1. These lesions were very light, however, TABLE II.‖

Subinoculation Tests of Suspensions of Anterior Quadrants of Spinal Cord Enlargements of Rhesus B430 (Preparalytic Poliomyelitis).

		Deaths or paralyses among inoculated animals			
	Virus Dilution	Rt. Cervical	Lt. Cervical	Rt. Lumbar	Lt. Lumbar
Mouse Tests	10^{-1}	11/16	7/8	0/8	0/8
	$10^{-1.5}$ and 10^{-2}	8/16	12/16	0/8	0/8
	$10^{-2.5}$ and 10^{-3}	4/14	4/14	0/8	0/8
	10^{-4}	1/16	1/16	0/8	0/8
Monkey Tests	10^{-1}			0/1	0/1

‖ The mouse titrations were part of a larger study of virus levels in infected monkeys being carried out in collaboration with Dr. Mary Cumberland. In this study it was found that intracerebral inoculation in rhesus monkeys was a more sensitive test for the presence of Lansing virus in monkey cord than was mouse inoculation. The monkeys shown in this table were inoculated intrathalamically and killed after three weeks. Sections of cord and brain were examined and found normal. Not enough material was available for additional monkey tests.

and sparse at C8 and T1. Neuronal changes were found only on the right side in segment C7. These consisted mostly of various degrees of chromatolysis with only about 4% of all cells on the left side destroyed.

Complete serial sections of the lumbar cord segments L3 to L7, and S1, were stained and examined. No lesions of poliomyelitis were found.

Every 20th section of the brain was stained and examined. A typical poliomyelitic distribution of moderate to severe perivascular and focal infiltrative lesions and of neuronal destruction was found. A predominance of severe lesions was observed on the right side of the brain stem, especially in globus pallidus, subthalamic centers, midbrain tegmentum, reticular formation, and vestibular nuclei. Lesions were not observed in the left cerebral cortex or thalamus.

Summary of Rhesus 959. This animal was of interest in that the only preparalytic symptoms noted were fever, and spasticity and increased knee tendon reflex of the right leg. Lesions were absent in the lumbar cord, and in the left motor cortex, but were severe on the right side of the brain stem, especially in the globus pallidus, subthalamic centers, midbrain tegmentum, reticular formation, and vestibular nuclei. If cortical involvement had been responsible for spasticity of the right leg, lesions would have been expected on the left side, but none were found there. The right-sided unilaterality of spasticity in this case is suggestively associated with ipsilateral predominance of lesions in the brain stem.

Rhesus B430. This was an adolescent animal in good condition.

Aug. 30, 1945. Under nembutal anaesthesia a trephine hole was prepared over the central sulcus in the midline, and 0.4 cc of a 5% rhesus cord suspension containing the Lansing strain of virus was inoculated into each dorsal thalamus.

Sept. 4. Rectal temperature had risen to 105°F. from a baseline of about 104°.

Sept. 5. The animal now was very awkward and stiff in running and climbing, and had marked head tremor. Palpation revealed that arms and legs were very strong, but very resistant to passive manipulation. All muscle groups appeared to be tense, but especially the forearm flexors and the hamstring muscles showed rigidity of the clasp-knife type. Tendon reflexes were exaggerated.

Under ether anaesthesia, the animal was exsanguinated from the femoral arteries. With sterile precautions, the cervical and lumbar enlargements of the spinal cord were removed separately for virus assay. The animal was then perfused through the aorta with 10% formalin containing 1% acetic acid. The brain and remainder of the spinal cord were removed for histological study.

Results of Virus Assays. Right and left anterior quadrants of cervical and lumbar enlargements were prepared separately as 10% aqueous suspensions. Subinoculations were done in mice and in rhesus monkeys, with the results seen in Table II.

Histopathological Findings. Segments of cord above and below the cervical block removed for virus, of various thoracic levels, and of the upper lumbar cord were imbedded in paraffin, sectioned serially at 15 μ, stained with gallocyanin, and examined. Cord from C1 to C3 contained numerous lesions of poliomyelitis, including perivascular and diffuse leucocytic infiltration, and chromatolysis and phagocytosis of nerve cells. Sections of cord at T2, T3, T5, T9, T11, T12, L1, and L2 were quite free of any lesions of poliomyelitis.

The brain was imbedded in celloidin and sectioned at 60 μ. Every 20th section was stained with gallocyanin and examined. A typical distribution of severe focal and diffuse infiltrative lesions and of neuronal destruction was found. Neuronal destruction and inflammatory changes were particularly severe in the reticular formation, especially in the pons region, and in the vestibular nuclei. Severe changes were also present in the midbrain tegmentum, in the fields of Forel, and in the substantia nigra. Lesions in the above-mentioned centers were roughly equal on both sides, but in the forebrain there was a predominance of lesions of moderate intensity in the left dorsal thalamus and in the left pre- and postcentral cortex. Lesions in the right thalamus and cortex were very few in number, and in fact absent in most sections examined.

Summary of Experiment B430. The failure to obtain virus from the lumbar enlargement or to find lesions in the thoracic or upper lumbar cord, whereas both lesions and virus were present in the cervical cord, is sufficient evidence that virus activity had not yet reached the lumbar cord in this animal.

Discussion. The evidence cited in this report indicates that neither virus activity nor lesions in the spinal cord are necessary pathogenetic factors for the production of spasticity in acute poliomyelitis. The marked spasticity seen in the legs of the monkeys described, in the absence of pathological changes in the lumbosacral cord, must be attributed to the pathological changes present in the brain or cervical region of the spinal cord. Although the lesions in the cervical cord could have affected the long propriospinal neurons connecting with motoneurons in the lumbar cord, this is doubtful since the lesions in the cervical region were quantitatively very mild. Severest damage in the brain in primate poliomyelitis including the greatest amount of nerve cell destruction most often occurs in the reticular formation of the hindbrain and in the vestibular centers. These centers are connected with motoneurons of the spinal cord either directly or by way of internuncial neurons, as is well known.[13] Magoun has made the interesting observation that stimulation of the bulbar reticular formation can have a generalized inhibiting effect on motor activity induced reflexly or by brain stem or cortical mechanisms,[14] and Wagley[15] has recently introduced evidence for the production of spasticity in rhesus monkeys by reticulospinal tract section.¶ These findings suggest that it is the severe lesions in the reticular formation in acute poliomyelitis which may be at least in part responsible for generalized spasticity, because of destruction of many "inhibitory" neurons. It is also of interest that all of the animals described in this account had almost fully developed pathological changes in the brain before limb paralysis was manifest.

---

[13] Lloyd, D. P. C., *J. Neurophysiol.*, 1941, **4**, 115.

[14] Magoun, H. W., *Science*, 1944, **100**, 549.

[15] Wagley, P. F., *Bull. Johns Hopkins Hosp.*, 1945, **77**, 218.

¶ The cells of origin of this tract are the large neurons scattered in the central part of the reticular formation from the level of the superior colliculus caudalward.[16] In a rhesus monkey prepared by Dr. Howard A. Howe and surviving 8 days after hemisection of the spinal cord at the first cervical segment, I found extensive and severe chromatolysis in these cells. The chromatolysis was bilateral but predominantly ipsilateral. It was estimated from counts made of every twentieth section of a 30 μ series, that the number of reticulospinal neurons on each side was of the order of one thousand. It is likely that these cells are part of a chain of neurons which form at least part of the extrapyramidal pathways from motor cortex to spinal cord (see review of Hines[17]).

[16] Papez, J. W., *J. Comp. Neur.*, 1926, **41**, 365.

[17] Hines, M., *Biol. Rev.*, 1943, **18**, 1.

The fact that spasticity can be produced without lesions being present in the spinal cord does not preclude the possibility, suggested by Kabat and Knapp,[8,9] that lesions in the internuncial neurons of the spinal cord can produce a similar effect under certain circumstances. As Lloyd[13] emphasizes, certain internuncial neurons in the cord are an integral part of the mechanism of reticulospinal and propriospinal action on the motoneurons. Kabat and Knapp have introduced as evidence in this connection the inconclusive observation that most spinal cords from fatal human cases have lesions in the intermediate columns where the internuncial neurons presumably are predominantly present, and that some cases have internuncial lesions with "relatively normal anterior horn cells." Unless serial sections of several segments at the involved level are examined, which was not implied, such a statement is necessarily inaccurate since lesions in the spinal cord may be quite spotty in character. For example, sections separated by only a millimeter or less may show quite different degrees of involvement of anterior, intermediate, or posterior cell columns.[10] It is therefore obvious that only a quantitative study of the degree of neuron destruction in the spinal cord centers at various levels, based on a detailed study of serial sections, and correlated with symptoms referable to affected levels in each case, can yield anything more than suggestive inferences in a problem of this kind. On the other hand, the evidence from preparalytic animals, as we have noted before,[18] can at times give a clear-cut answer on problems of pathogenesis because in such animals the pathological picture is in process of development and much less difficult of interpretation than that in fatal cases. The absence of lesions or detectable virus activity in the lumbosacral cord in the preparalytic period of animals already showing spasticity of leg muscles, is an unusually good example of this.

Summary. Evidence is presented which demonstrates that neither virus activity nor lesions in the spinal cord are necessary pathogenetic factors for the production of the spasticity of acute poliomyelitis. In 2 rhesus monkeys inoculated intracerebrally with poliomyelitis virus and killed in the preparalytic period when marked spasticity was present in the muscles of the legs, as well as elsewhere, no lesions were present in complete serial sections of the lumbosacral cord. In an additional similar case it was also found that no virus was present in the lumbosacral cord. In all cases severe lesions were already present in most of the brain centers usually involved, and neuronal destruction was especially severe in the midbrain tegmentum, reticular formation of the hindbrain, and in the vestibular nuclei. It is concluded that lesions in the brain alone can produce the spasticity of acute poliomyelitis. The pathological origin of this symptom is discussed, and evidence cited for the possible causative role of lesions in brain stem centers, especially the reticular formation.*

---

[18] Bodian, D., and Howe, H. A., *Brain*, 1940, **63**, 135.

* While this paper was in press, Dr. Isabel Morgan reminded the author that Bodian and Howe[18] in 1940 (p. 152) described two relevant rhesus monkeys killed in the preparalytic stage following intrasciatic virus inoculation. When killed these animals had generalized prodromal muscle tenseness, although lesions had not progressed rostrally beyond the midbrain tegmentum in one case or the hypothalamus in the other, so that no cortical lesions were found. A resurvey of serial sections of the central nervous system of both of these cases showed that lesions were absent in vestibular nuclei, very light in the cervical cord, and very severe in the reticular formation of the pons region.

15263 P

Folic Acid Therapy in Macrocytic Anemias of Infancy.

Wolf W. Zuelzer, and F. N. Ogden. (Introduced by Icie G. Macy.)
From the Anemia Clinic of the Children's Hospital of Michigan, Detroit, and the Department of Pediatrics, Wayne University College of Medicine.

The clinical and hematological picture of macrocytic anemias of infancy has not been sharply defined. Favorable responses to liver extracts have been reported sporadically. In studying infants with macrocytic anemia characterized by a megaloblastic type of erythropoiesis we treated a number of cases with folic acid in the form of a concentrate prepared from liver, "L. Casei factor," or with synthetic folic acid.* This report presents the results obtained with the 4 patients for whom the data have been calculated. A full report on the entire group, presenting the morphologic criteria used in determining this type of anemia, with a review of the literature, is now in preparation.

The patients were white infants, ranging from 2 to 12 months in age, one male and 3 females. All came from average homes and had been well cared for. They had received good diets, supplemented by cod liver oil and orange juice in adequate amounts. One patient (Case 3), born prematurely, had weighed 4 pounds at birth. The others had been full-term babies.

The clinical picture was not characteristic. All patients had a short history of vomiting, anorexia and pallor, as a rule preceded by signs of an upper respiratory infection and fever, which usually persisted. Three patients had had mild diarrhea; 2 had been treated with sulfadiazine.

The pertinent findings of the physical examination on admission to the hospital were marked pallor, usually slight cardiac enlargement and a soft systolic murmur. The spleen was moderately enlarged and firm.

After admission, daily complete blood counts were made. Hemoglobin was determined as oxyhemoglobin with the Evelyn photoelectric colorimeter. Reticulocyte counts were obtained by the "dry" method. Bone marrow was aspirated from the lower end of the femur on admission and at intervals during the period of observation. The liquid marrow was heparinized, centrifuged and the volumetric pattern determined. Smears of the buffy coat were stained with modifications of the Romanowsky stain and differential counts of at least 500 nucleated cells were made.

All patients had severe macrocytic anemia, accompanied by moderate to marked diminution of the blood platelets. Case 2 showed mild leukopenia and neutropenia. A few nucleated red cells were invariably present in blood smears and often suggested a megaloblastic origin. The bone marrow was characterized by a megaloblastic type of erythropoiesis and changes in the granulocytes closely resembling, but not identical with, the marrow lesion in Addisonian pernicious anemia. Volumetric readings indicated moderate hypoplasia rather than hyperplasia.

In 3 cases blood transfusions were deemed necessary. These were followed by temporary increases in hemoglobin and red blood cells. Before treatment was started the hemoglobin level had begun to fall. Treatment with folic acid was not begun until preliminary observation had established the absence of a spontaneous reticulocytosis.

The response to folic acid was characterized by a rise in the reticulocyte count on the third or fourth day, which reached a maximum on the sixth or seventh day and was followed by steady rises in the hemoglobin values and red blood cell counts. The curves were virtually identical with those obtained in pernicious anemia following adequate treatment with purified liver extract. (Fig. 1). The hemoglobin tended to reach normal levels in approximately 3 weeks. The platelets became plentiful within one week. Character-

---

* Both preparations were made available through the courtesy of the Lederle Laboratories.

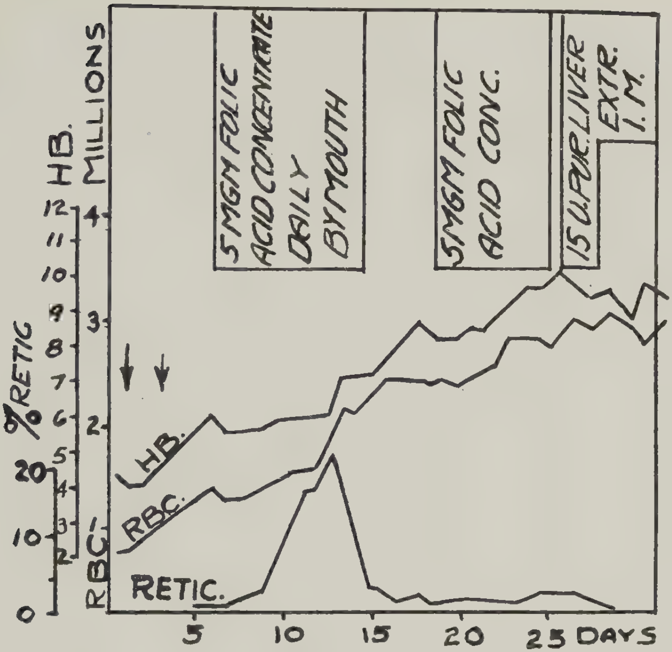


Fig. 1.

Case I illustrating the typical response to folic acid concentrate given orally.

istic changes in the bone marrow took place as early as the fourth day. Megaloblastic erythropoiesis yielded to a normoblastic pattern and the cellularity of the femoral marrow increased markedly.

With improvement in the blood picture, the clinical symptoms disappeared, the appetite improved and healthy color returned gradually. The spleen usually remained palpable for some time. A second course of folic acid or injections of purified liver extract produced no appreciable additional response.

We consider therapy with folic acid specific in this type of anemia because: (1) it produces an adequate reticulocyte response, followed by a marked, lasting improvement in the red blood counts and hemoglobin values; (2) equally important, the megaloblastic pattern of the bone marrow is transformed into a normoblastic one. Analogous changes occur in Addisonian pernicious anemia after specific therapy with liver extract[1,2] and, recently, have been shown in nutritional macrocytic anemias of adults.[3,4]

The genesis of this disturbance which is specifically influenced by folic acid remains obscure.

Conclusion. Certain macrocytic anemias of infancy, characterized by megaloblastic erythropoiesis respond specifically to folic acid.

---

[1] Jones, O. P., in Downey, H., *Handbook of Hematology*, Vol. 3, p. 2045, Paul H. Hoeber, Inc., New York, 1938.

[2] Naegeli, O., *Wien Klin. Wochnsch.*, 1935, **48**, 225.

[3] Spies, T. D., Vilter, C. S., Koch, N. B., and Caldwell, M. H., *Southern Med. J.*, 1945, **38**, 707.

[4] Moore, C. V., Vilter, R., Minnich, V., and Spies, T. D., *J. Lab. Clin. Med.*, 1944, **29**, 1226.

15264

Effect of Acetic, Monochloroacetic, Dichloroacetic, and Trichloroacetic Acids on Oxygen Consumption of Mouse Liver.

EDWIN P. LAUG. (Introduced by O. Garth Fitzhugh.)

From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

From acute oral toxicity data in mice, rats and guinea pigs[1] it has been shown that monochloroacetic acid is from 25 to 40 times as toxic as acetic acid and its di- and trichloro derivatives. It has not been determined from these studies what causes death nor have chronic feeding experiments indicated any clearly defined functional or organic derangements. While the effect of monoiodoacetic acid on the glycolytic and oxidative functions of tissues has been amply demonstrated, such information on monochloroacetic acid is at best somewhat meager. It has been recorded that monochloroacetic acid has cholagogic action[2] and Lundsgaard[3] states that in comparison to monoiodoacetic acid, monochloroacetic acid inhibits yeast fermentation only feebly. The present experiments were undertaken to determine what effect, if any, mono-, di- and trichloroacetic acid would have on the respiration of liver tissue.

Method. Liver slices from well fed young adult mice were used. Oxygen consumption was measured by the standard Warburg procedure at 37.5°C. Acetic, mono-, di-, and trichloroacetic acids were neutralized and diluted in phosphate buffered glucose-Ringer, so that the final concentration in contact with the liver slice ranged from 0.03M to 0.00005M. The pH of all solutions was adjusted to 7.4.

Results. Fig. 1 shows in graphic form the typical effects of mono-, di-, and trichloroacetic acids at 0.0005M concentration on the respiration of mouse liver slices. Respiration is promptly and precipitously reduced by

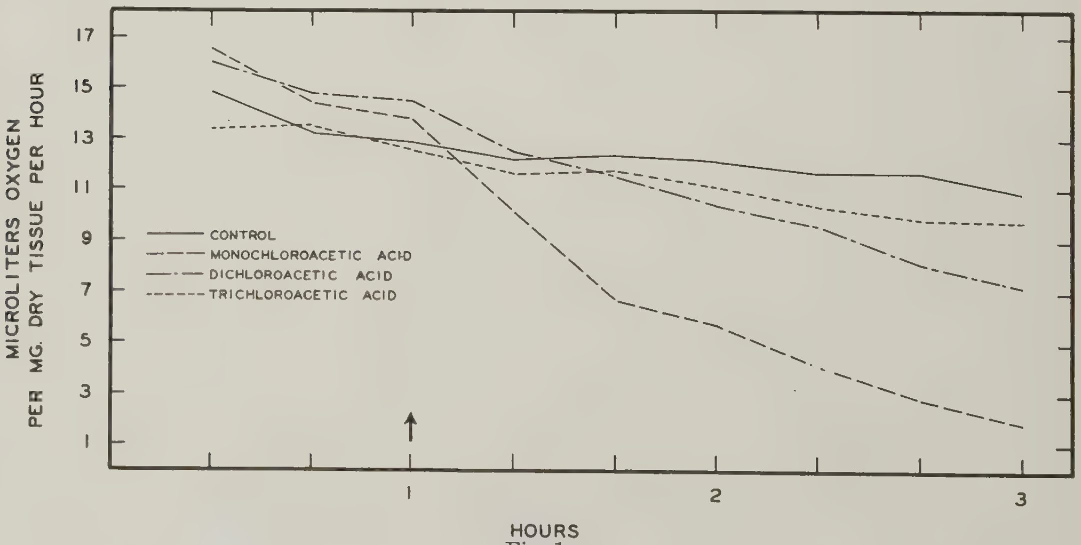


Fig. 1.

Effect of 0.005M mono-, di-, and trichloroacetic acids on oxygen consumption of mouse liver slices. Temp. 37.5° C, pH 7.4.

---

[1] Woodard, G., Lange, S. W., Nelson, K. W., and Calvery, H. O., *J. Indust. Hyg. and Tox.*, 1941, **23**, 78.

[2] Chabrol, E., Charonnat, R., Maximin, M., and Waitz, R., *Compt. rend. Soc. Biol.*, 1931, **106**, 17.

[3] Lundsgaard, E., *Biochem. Z.*, 1932, **250**, 61.

monochloroacetic acid, and within 2 hours almost completely abolished. The effect of di- and trichloroacetic acids, while clearly defined, is much weaker and more delayed. It is important to note that here, as at all other concentrations studied, the order of decreasing effect on respiration is: monochloroacetic acid > dichloroacetic acid > trichloroacetic acid. Acetic acid in the range of 0.01M to 0.001M showed no effect.

Within the range of 0.001M to 0.03M, the depression of respiration by mono- and dichloroacetic acids is maximal; there is no indication of a progressively increasing effect with increasing concentration. Within the range of 0.001M to 0.00005M there is a gradually decreasing effect, which ceases below 0.00005M for both acids. This is a concentration of the order of 5 to 10 μg per milliliter of Ringer solution. At a concentration below 0.005M no effect of trichloroacetic acid has been noted, and at 0.01M evidences of tissue coagulation have been seen.

Summary. At the concentrations studied monochloroacetic acid produces a marked depression in the respiration of mouse liver slices. The order of decreasing effect of the 3 acids is: monochloroacetic > dichloroacetic > trichloroacetic. Acetic acid has no effect.

15265

Electrophoretic Analysis of Kala-azar Human Serum. Hypergammaglobulinemia Associated with Seronegative Reactions for Syphilis.*

G. R. COOPER, CHARLES R. REIN, AND J. W. BEARD.

From the Department of Surgery, Duke University School of Medicine, Durham, N. C., and the Division of Serology, Army Medical School, Army Medical Center, Washington, D. C.

It is generally recognized that nonspecific positive reactions in tests for syphilis may occur with human sera from normal individuals[1,2] and from individuals affected with certain diseases[3-5] and conditions not associated with syphilis. The belief has arisen among serologists that nonspecific positive reactions are associated with hyperglobulinemia, and the presence of a high serum protein or globulin content has been suggested[6] as a criterion of the nonspecificity of a positive reaction.

The disease, kala-azar, is characterized in part by hyperglobulinemia[7] associated with the presence in high concentration of abnormal euglobulin, which gives rise to the formolgel[8] reaction and a cold-precipitable pathological globulin.[9] In this disease, also, there has been reported[10] the occurrence of nonspecific positive reaction in tests for syphilis. Recently, in connection with a comparative study

---

* The work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Duke University.

[1] Eagle, H., *Am. J. Syph.*, 1941, **25**, 7.

[2] Mohr, C. F., Moore, J. E., and Eagle, H., *Arch. Int. Med.*, 1941, **68**, 898.

[3] Mohr, C. F., Moore, J. E., and Eagle, H., *Arch. Int. Med.*, 1941, **68**, 1161.

[4] Hazen, H. H., Parran, T., Sanford, A. H., Senear, F. E., Simpson, W. M., and Vonderlehr, R. A., *Internat. J. Leprosy*, 1936, **4**, 315.

[5] Davis, B. D., *Medicine*, 1944, **23**, 359.

[6] Cardon, L., and Atlas, D. H., with the assistance of Aron, E., Brunner, M. J., Teitelman, S. L., and Bunata, J., *Arch. Dermat. and Syph.*, 1942, **46**, 713.

[7] Lloyd, R. B., and Paul, S. N., *Indian J. Med. Res.*, 1928-1929, **16**, 203.

[8] Napier, L. E., *Indian J. Med. Res.*, 1921-1922, **9**, 830.

[9] Wertheimer, E., and Stein, L., *J. Lab. and Clin. Med.*, 1944, **29**, 1082.

[10] Greval, S. D. S., Gupta, P. C. S., and Napier, L. E., *Indian J. Med. Res.*, 1939-1940, **27**, 181.

TABLE I.
Percentage Distribution, Concentration and Mobilities of the Components of Normal and Kala-azar Human Sera.

				Components				
					Globulins			
	Sample	Serum	Electrophoresis Solution	Albumin A	α_1	α_2	β	γ
% Distribution	477			21.7	3.0	3.4	7.8	64.1
	499			50.3	2.3	11.6	7.4	28.4
	Normal*			60.0	3.6	9.1	12.5	14.8
Protein Concentration gm per 100 ml	477	12.1	2.88	2.62	0.36	0.41	0.95	7.76
	499	8.65	2.58	4.35	0.20	1.00	0.64	2.46
	Normal*	7.23	—	4.34	0.26	0.66	0.90	1.07
Mobility $-\mu \times 10^5$ $cm^2 sec^{-1} volt^{-1}$	477			5.88	5.00	3.94	2.79	0.58
	499			5.98	5.04	3.91	3.08	0.62
	Normal*			6.08	5.04	3.93	2.97	1.27

* Avg. values of 13 normal human sera.[11]

of syphilitic and nonspecific positive sera,[11] an opportunity was afforded for the examination of a serum from each of 2 patients with kala-azar. Electrophoretic analyses were made of the distribution, concentration and mobilities of the serum protein components, and the sera were thoroughly studied with a battery of serodiagnostic tests for syphilis. The results of these electrophoretic and serological studies are reported in the present paper.

Materials and Methods. The sera[†] were obtained from 2 patients in whom the diagnosis of kala-azar was established by the recovery of Leishman-Donovan bodies by puncture of the spleen and sternal marrow. One, Serum 477, was from a 23-year-old colored male, C.W.Y., who became ill February 28, 1944. The serum proteins were consistently elevated to levels reaching 12 g per 100 ml. The blood for electrophoretic analysis was drawn September 8, 1944, while the patient was still clinically ill. The second patient, Serum 499, was a 24-year-old white male, A.W.F., who had malaria in the summer of 1943 and typhoid in January, 1944. The onset of kala-azar occurred in April, 1944. The total serum protein and globulin contents were 6.7 and 3.35; 8.9 and 4.3; and 7.75 and 2.75 gm per 100 ml in July, August and October, respectively. The serum for the present studies was drawn on November 17, 1944, about 30 days after abatement of the disease.

Electrophoresis was carried out as previously described[11,12] at 1° in barbital buffer[13] at pH 8.6 and 0.1 ionic strength. The period of migration of Serum 477 was 234 minutes at 5.95 volts per cm and of Serum 499, 180 minutes at 6.42 volts per cm. The patterns representing the migrating boundaries were recorded by the method of crossed slits described by Svensson.[14]

The serum was prepared for electrophoresis by dilution with the barbital buffer to approximately 2% protein content and dialysis against frequently changed buffer at 2 to 8°. Protein concentrations were determined by the falling-drop procedure[15] standardized by Kjeldahl analyses.

Component analyses were made from measurements in photographic enlargements of the electrophoretic diagrams in which the components were separated by vertical lines

---

[11] Cooper, G. R., Craig, H. W., and Beard, J. W., in press.

[†] The sera and histories of the patients were provided through the generosity of Capt. James Liebmann, M. C., A.U.S., Walter Reed General Hospital, Washington, D.C.

[12] Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1942, **142**, 193.

[13] Longsworth, L. G., *Chem. Rev.*, 1942, **30**, 323.

[14] Svensson, H., *Kolloid Z.*, 1940, **90**, 141.

[15] Barbour, H. G., and Hamilton, W. F., *J. Biol. Chem.*, 1926, **69**, 625.

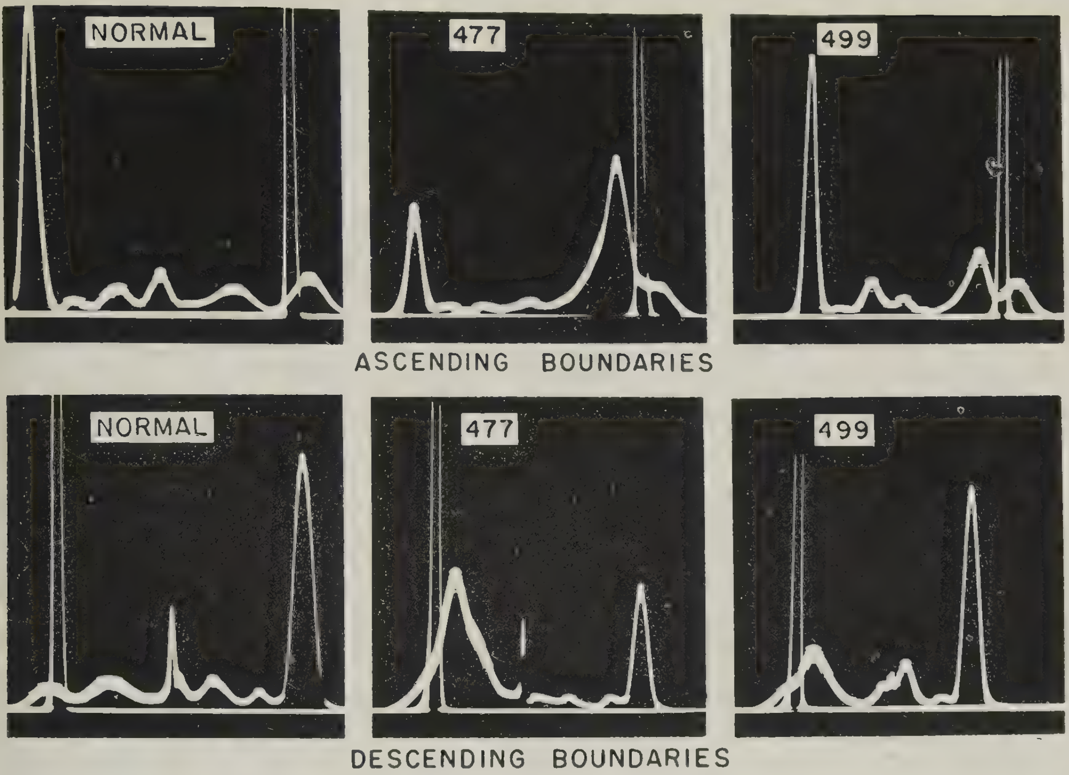


Fig. 1.

Electrophoretic patterns of human serum from a normal individual and from 2 patients diseased with kala-azar.

to the base at the minima between the peaks.[16] The respective areas were bounded by the mid-line of the curve above and the mid-line of the base below. The mobilities of the various components were calculated from the distances measured from the starting point of migration to the maximum ordinates of the respective peaks.[13] Calculations were made for both ascending and descending sides and the values given in Table I are the average of the 2 sets of data.

The sera of both patients were subjected to serologic examinations with a battery of 6 serodiagnostic tests, including the Kline diagnostic, Kline exclusion, Boerner-Jones-Lukens, Mazzini, Kahn and Kolmer procedures. The tests were made on other sera drawn in August, September and October of 1944 in addition to the 2 sera on which the electrophoretic studies were made. Further, the blood specimen, Serum 477, drawn from patient C.W.F. on September 8, 1944, was also subjected to chemical fractionation by Dr. Hans Neurath, Duke University School of Medicine, and each fraction, the albumin and the GI, GII and GIII globulins, was tested with the 5 flocculation procedures enumerated above. Formol-gel tests[8] were made on both of the sera.

Results. Electrophoretic patterns of the ascending and descending boundaries of the kala-azar sera and of a normal[11] serum for comparison are reproduced in Fig. 1. The pattern of Serum 477 reveals a γ globulin peak much greater in height and area than the albumin peak. In addition, there is pronounced skewing of the γ globulin peak to the slow side and diffuseness of the curve on the fast side. The percentage distribution and actual concentration of the components of Serum 477 are given in Table I. It is seen that the γ globulin fraction was about 7 times the normal value,[11] while the albumin was less than one-half the normal. The other electrophoretic fractions showed insignificant deviation from the concentrations observed in normal serum.

---

[16] Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, 1939, **69**, 119.

The mobilities of the proteins of Serum 477 (Table I) were the same as those of the components of normal serum,[11] except in the instance of the γ globulin fraction. The mobility of this fraction was outside the limits of the mobility usually associated with normal human γ globulin.[11] The diffuseness in the curve in the early part of the γ peak indicates the presence of the normal γ component.

Similar abnormalities but of less degree were observed in the proteins of Serum 499. The γ globulin peak was sharply heightened and skewed to the slow side of the γ globulin region. There was little suggestion of disturbance of the curve corresponding to the region of the normally behaving γ globulin. In percentage composition (Table I) the γ globulin fraction was double the normal, and the albumin and β globulin fractions were slightly less than normal. The actual concentrations of the components (Table I) showed that no significant changes from the normal had occurred except in the γ globulin fraction. The β anomaly peak[13] was absent. Mobilities of the components were within the normal range except that of the γ globulin fraction, which was found to be the same as in Serum 477.

The serodiagnostic tests on all of the serum samples investigated were uniformly negative and so, too, were the tests of the albumin and GI, GII and GIII globulins obtained by chemical fractionation. The sera, likewise, were negative to the formol-gel test.

Discussion. The abnormalities of the γ globulin component of kala-azar sera differ greatly from those of the serum of patients with other diseases involving the liver and spleen.[17,18] In syphilis,[11] rheumatic fever,[19] sarcoidosis,[20,21] tuberculosis,[22] as well as other pathological states,[23–25] the rise in γ globulin is associated with the entire normal γ globulin region. The findings indicate the presence in human kala-azar serum of a γ globulin component not previously observed in human serum. A γ globulin[26] component of low mobility has been observed, however, in the serum of rabbits hyperimmunized with Type 1 pneumococci.

The possible relation between the abnormal γ globulin and the globulins giving the formol-gel reaction[8] or precipitating in the cold[9] is subject only to surmise. Since the formol-gel test was negative, the abnormal γ globulin could not have been representative of the whole of the material responsible for this reaction. The cold-precipitable euglobulin, which may persist after recovery, may have contributed to the γ globulin. The electrophoretic abnormalities, pronounced during the course of the disease and still present in the convalescent serum, may prove on further investigation to be unique with kala-azar and of greater diagnostic significance than the formol-gel test or cold precipitation.

The nonspecific positive reactions in tests for syphilis seen in a variety of nonsyphilitic diseases and conditions may be due: to the presence of antibody-like substances similar to the antibodies produced in syphilitic disease; to an increase or alteration of some chemical substance or substances in the blood; or to an increase or alteration of the seroglobulin fraction. Cardon and Atlas[6] investigated a series of 34 patients with hyperproteinemia, of which 8 gave nonspecific positive reactions for syphilis. These au-

---

[17] Luetscher, J. A., Jr., *J. Clin. Invest.*, 1940, **19**, 313.

[18] Gray, S. J., and Barron, E. S. G., *J. Clin. Invest.*, 1943, **22**, 191.

[19] Rutstein, D. D., Clarke, F. H., and Taran, L. M., *Science*, 1945, **101**, 669.

[20] Seibert, F. B., and Nelson, J. W., *Am. Rev. Tuberc.*, 1943, **47**, 66.

[21] Fisher, A. M., and Davis, B. D., *Bull. Johns Hopkins Hosp.*, 1942, **71**, 364.

[22] Seibert, F. B., and Nelson, J. W., *J. Biol. Chem.*, 1942, **143**, 29.

[23] Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., *J. Exp. Med.*, 1939, **70**, 399.

[24] Luetscher, J. A., Jr., *J. Clin. Invest.*, 1941, **20**, 99.

[25] Shedlovsky, T., and Scudder, J., *J. Exp. Med.*, 1942, **75**, 119.

[26] van der Scheer, J., Bohnel, E., Clarke, F. H., and Wyckoff, R. W. G., *J. Immunol.*, 1942, **44**, 165.

thors believe that blood protein studies should be instituted when a nonspecific positive reaction is suspected and that care should be taken in the interpretation of a positive serologic reaction for syphilis in the presence of conditions with hyperproteinemia and hyperglobulinemia. Proven syphilitics may also show[11] a hypergammaglobulinemia and patients with kala-azar, as shown in the present work, and sarcoidosis[27] may have hyperglobulinemia associated with negative serologic reactions for syphilis. The mere presence or absence of hyperproteinemia, hyperglobulinemia or hypergammaglobulinemia is not sufficient to prove or disprove the specificity of positive serologic reactions for syphilis.

Summary. The electrophoretic patterns of 2 human kala-azar sera revealed the presence in high concentration of a unique, abnormal component migrating with the γ globulin of the slowest mobility. The kala-azar sera, negative to serological tests for syphilis, provide instances which show that the presence of hyperproteinemia, hyperglobulinemia or hypergammaglobulinemia cannot be used to prove or disprove the specificity of positive serologic reactions for syphilis.

---

[27] Cooper, G. R., Rein, C. R., and Beard, J. W., unpublished observations.

15266

Inactivation of Glutamic-Aspartic Transaminase by Irradiation.*

F. SCHLENK, A. FISHER, AND E. E. SNELL.

From the M. D. Anderson Hospital for Cancer Research, University of Texas, Houston, and the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

Glutamic-aspartic transaminase is an enzyme which catalyzes the reaction: 1 (+) glutamic acid + oxaloacetic acid $\rightleftarrows$ α-ketoglutaric acid + 1 (—) aspartic acid. The prosthetic group of this enzyme is a derivative of vitamin B_6,[1,2] probably identical with codecarboxylase.[3,4] Since pyridoxine, pyridoxal and pyridoxamine are very sensitive to light,[5,6,7] it was expected that transaminase preparations would also be inactivated by irradiation.

It was found that sunlight destroys the enzymatic activity at about the same rate as it inactivates the vitamin B_6 present in the preparations (Table I). The destruction by ultraviolet light is much more rapid (Table II). The effect of X-rays on transaminase was also investigated. Vitamin B_6 has been considered as a remedy for X-ray sickness.[8,9] It was found that X-ray irradiation in therapeutic and higher doses has no effect on transaminase activity. Therefore, the possible therapeutic value of vitamin B_6 in this condition cannot be explained in terms of a restoration of inactivated transaminase.

Experimental Procedures. For the determination of transaminase activity the procedures developed by Cohen[10] were used. The enzyme was purified according to the

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Reported at the Dallas Meeting, Southwestern Section, on October 20, 1945.

[1] Schlenk, F., and Snell, E. E., *J. Biol. Chem.*, 1945, **157**, 425.

[2] Schlenk, F., and Fisher, A., *Arch. Biochem.*, 1945, **8**, 337.

[3] Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1945, **161**, 311.

[4] Green, D. E., Leloir, L. F., and Nocito, V., *J. Biol. Chem.*, 1945, **161**, 559.

[5] Hochberg, M., Melnick, D., Siegel, L., and Oser, B. L., *J. Biol. Chem.*, 1943, **148**, 253.

[6] Hochberg, M., Melnick, D., and Oser, B. L., *J. Biol. Chem.*, 1944, **155**, 129.

[7] Cunningham, E., and Snell, E. E., *J. Biol. Chem.*, 1945, **158**, 491.

[8] Maxfield, J. R. Jr., McIlwain, A. J., and Robertson, J. E., *Radiology*, 1943, **41**, 383.

[9] Gendreau, J. E., and Lafleur, L., *Union med. du Canada*, 1944, **73**, 666.

[10] Cohen, P. P., *J. Biol. Chem.*, 1940, **136**, 565.

TABLE I.
Inactivation of Glutamic-Aspartic Transaminase by Sunlight.

	Irradiated				Dark controls			
		Vitamin B_6 present as determined with*				Vitamin B_6 present as determined with*		
Time (hrs)	Transaminase activity %	*L. casei* γ per g	*S. fecalis* R γ per g	*Sacc. carlsbergensis* γ per g	Transaminase activity %	*L. casei* γ per g	*S. fecalis* R γ per g	*Sacc. carlsbergensis* γ per g
0	30.5	67	305	95	30.5	67	305	95
1	14.9	24	40	31	29.0	47	267	75
2	(1.9)	19	39	25	29.0	56	276	82
3	6.1	11	22	18	31.4	50	305	79

* Pyridoxal was used as standard with *L. casei*, pyridoxamine with *S. fecalis* R, and pyridoxine with *Sacc. carlsbergensis*.

method outlined earlier[2] which yields solutions with no absorption in the visible part of the spectrum.

Irradiation by Sunlight. For irradiation by sunlight large open Petri dishes were placed in an icebath. The light path was 0.4 cm; irradiation was from 1 P.M. to 4 P.M., May 14, 1945; 29° North latitude. At the times given in Table I samples were withdrawn for the determination of transaminase and for the microbiological assay of the B_6 vitamins.[11]

Microbiological assay was carried out following 15 minutes autoclaving of the sample with N/10 sulfuric acid. No attempt at differential assay[12] for various members of the vitamin B_6 group was made, since the relative activity of the coenzyme for the various organisms and the conditions which effect its complete hydrolysis and release from the protein molecule with which it is associated are at present unknown.[4] The figures given in Table I represent the total vitamin B_6 per gram of protein as indicated by assay with each organism against the appropriate standard.

Irradiation by Ultraviolet Light. For ultraviolet irradiation a quartz mercury lamp (General Electric Co., Model F; 400 watt) was used. The distance between the lamp and the solution was 45 cm. Otherwise the procedure outlined above was followed. For comparison solutions of pyridoxine, pyridoxal, and pyridoxamine (10 γ per ml) were irradiated under the same conditions. The destruction was determined using the colorimetric reaction of Swaminathan[13] in the modification of Bina, Thomas, and Brown.[14]

The inactivation as recorded in Tables I and II is not due to hydrogen peroxide. The hydrogen peroxide concentration in distilled water irradiated by ultraviolet light under the same conditions for 30 minutes remained below 6×10^{-5} moles per liter. We found

---

[11] Snell, E. E., and Rannefeld, A. N., *J. Biol. Chem.*, 1945, **157**, 475.

[12] Snell, E. E., *J. Biol. Chem.*, 1945, **157**, 491.

[13] Swaminathan, M., *Nature* (London), 1940, **145**, 780.

[14] Bina, A. F., Thomas, J. M., and Brown, E. B., *J. Biol. Chem.*, 1943, **148**, 111.

TABLE II.
Inactivation of Transaminase and of Vitamin B_6 by Ultraviolet Light.

Irradiation (min.)	Transaminase activity %	Vitamin B_6 compounds Pyridoxal γ	 Pyridoxamine γ	 Pyridoxine γ
0	37.2	40.0	40.0	40.0
5	10.3	8.0	18.5	14.3
10	4.3	2.1	9.8	8.9
15	1.9	1.2	5.9	4.3
30	<1	0.6	2.9	0.8

that transaminase is rather resistant to hydrogen peroxide. The stability of the members of the vitamin B_6 group has been reported earlier.[7] The inactivated enzyme was not reactivated by addition of pyridoxine, pyridoxal, pyridoxamine, or boiled tissue extract.

Irradiation by X-rays. The conditions were as follow:† 200 K.V.; 16 milliamp; 50 cm distance; filter, 2.5 mm. Al inherent in tube, none added. Since no appreciable inactivation of transaminase (Table III) was observed, no vitamin B_6 determinations were carried out.

TABLE III.
X-Ray Irradiation of Transaminase.

Irradiation r units	Transaminase activity %
0	39.7
500	36.2
1,500	38.4
4,000	35.8
7,000	39.1
10,000	34.5

Summary. Glutamic-aspartic transaminase is inactivated by sunlight and by ultraviolet light. Destruction of enzymatic activity by these agents parallels the destruction of vitamin B_6, a derivative of which is present as the prosthetic group of the enzyme.[1-4] The irradiated enzyme is not reactivated by addition of pyridoxine, pyridoxal, pyridoxamine or boiled tissue extracts. Irradiation with X-rays leaves the enzyme intact.

---

† The authors are indebted to Dr. T. G. Russell for collaboration.

15267

Biological Activity and Metabolism of d, l-O-Heterobiotin in the Chick.*

P. R. MOORE, T. D. LUCKEY, C. A. ELVEHJEM, AND E. B. HART.

From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

O-Heterobiotin, an oxygen analog of biotin, has biotin-like activity for the chick[1] and for rats fed egg white.[1,2] Hofmann *et al.*[1] found the d,l analog 10% as active as d-biotin in curing egg-white toxicity in rats and approximately 6 and 20% as active in promoting growth in chicks when fed at different levels on a biotin deficient ration. Rubin *et al.*[2] found d,l-O-heterobiotin 5% as active as d-biotin in counteracting egg-white toxicity in the rat.

Since levels of 40 γ of d,l-O-heterobiotin

---

* Published with the approval of the Director of the Wisconsin Agricultural Experimental Station. Supported in part by grants from the Wisconsin Alumni Research Foundation and Swift and Company.

We are indebted to Merck and Company, Rahway, N. J., for crystalline vitamins; to Dr. S. H. Rubin and Dr. J. A. Aeschlimann of Hoffmann-LaRoche, Inc., Nutley, N. J. for O-heterobiotin; to Dr. B. L. Hutchings of Lederle Laboratories, Inc., for crystalline folic acid; to Abbott Laboratories, North Chicago, for haliver oil; to E. I.

TABLE I.

Activity and Storage of d,l-O-Heterobiotin* in the Chick.

Group No.	Supplement per 100 g biotin deficient basal	No. of survivors at 4 wks.	Avg. wt. at 4 wks.	No. of chicks with dermatitis at 4 wks.	d,l-O-Heterobiotin content of Liver	d,l-O-Heterobiotin content of Leg muscle
			g		γ/g	γ/g
1	None	11†	185	11	0.0	0.0
2	2.5 γ d-biotin	6	185	3		
3	5 γ ’’	3	205	0		
4	10 γ ’’	5	220	0		
5	20 γ ’’	11†	240	0	0.0	0.0
6	5 γ d,l-O-heterobiotin	6	180	4		
7	10 γ ’’	6	175	2		
8	20 γ ’’	6	195	0	0.3	0.0
9	200 γ ’’	6	205	0		
10	500 γ ’’	4	215	0	0.5	0.0
11	1000 γ ’’	6	210	0	5.3	0.2

* d,l-O-Heterobiotin is compared with d-biotin on a weight basis throughout this work. If only the d-form of the analog is active the values should be increased correspondingly.

† 12 chicks per group.

per 100 g of a biotin-deficient ration have given only partial responses in chicks[1] it was interesting to determine if higher levels could eventually give complete protection. At the same time it was possible to study the distribution of the analog in chick tissues when graded levels of the compound were included in the diet.

Day-old White Leghorn cockerels were maintained in cages with raised screen bottoms and heated by carbon filament lamps. The chicks were fed a purified ration having the following composition: dextrin 61 g, casein 18 g, gelatin 10 g, Salts V[3] 6 g, soy bean oil 5 g, 1(—) cystine 0.3 g, i-inositol 0.1 g, choline chloride 0.15 g, thiamine hydrochloride 0.3 mg, pyridoxine hydrochloride 0.4 mg, riboflavin 0.6 mg, Ca pantothenate 2 mg, nicotinic acid 5 mg, 2-methyl-1,4-naphthoquinone 0.05 mg, α-tocopherol 0.3 mg, p-aminobenzoic acid 5 mg, and crystalline folic acid 0.05 mg. Water and feed were given *ad libitum*, and each chick received weekly one drop of a vitamin A and D concentrate containing 1200 U.S.P. units of vitamin A and 120 A.O.A.C. units of crystalline vitamin D_3 per drop. After 3 days on the basal ration chicks within a 10 g weight range were divided into uniform groups of 6 and given supplements of d-biotin or d,l-O-heterobiotin in the basal ration. The feeding trials were terminated when the chicks were 4 weeks old and samples of liver and leg muscle were taken from 4 chicks in each group for O-heterobiotin analysis. O-Heterobiotin was determined by the differential microbiological assay of Luckey *et al.*[4]

The average weight at 4 weeks, incidence of dermatitis, and O-heterobiotin content of the tissues are summarized in Table I. A comparison of the relative effectiveness of d,l-O-heterobiotin and d-biotin in curing dermatitis indicates that the d,l analog is about ⅓ as active as d-biotin. However, in a comparison based on growth the activities varied inversely with the amount of analog fed. 20 γ of the d,l analog had an activity of approximately 20%. With higher levels the activity fell off rapidly and values from

---

du Pont de Nemours and Company, Inc., New Brunswick, N. J., for crystalline vitamin D_3; to Wilson and Company, Inc., Chicago, for gelatin; to Allied Mills, Inc., Peoria, Ill., for soy bean oil; and to Mr. J. Kamhi for technical assistance.

[1] Hofmann, K., McCoy, R. H., Felton, J. R., Axelrod, A. E., and Pilgrim, F. J., *Arch. Biochem.*, 1945, **7**, 393.

[2] Rubin, S. H., Flower, D., Rosen, F., and Drexter, L., *Arch. Biochem.*, 1945, **8**, 79.

[3] Briggs, G. M., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1943, **148**, 163.

[4] Luckey, T. D., Moore, P. R., and Elvehjem, C. A., in press.

0.5 to 3% were obtained. It is apparent that a level of d,l-O-heterobiotin as high as 1000 γ per 100 g of ration does not give a complete growth response in chicks on our biotin deficient ration and it appears that higher levels would not have afforded complete protection since the 1000 γ level actually gave no better response than the 500 γ level. This failure of O-heterobiotin to completely replace d-biotin for growth when it is highly active in the prevention of dermatitis indicates that the analog is capable of fulfilling only part of the function of d-biotin in the chick. On the other hand, the plateau in growth observed above the 500 γ level (compare group 11 to 10) suggests an inhibitory effect of the d,l analog on growth. In this connection, Luckey *et al.*[4] have observed an inhibitory effect of low levels of d,l-O-heterobiotin on the growth of *Streptococcus fecalis*. Further experimental data are required to clarify this point.

The analyses for d,l-O-heterobiotin indicate that this compound does not occur naturally in chicks receiving our biotin deficient diet alone or when supplemented with d-biotin. No appreciable accumulation of the d,l analog occurred in the livers of the chicks receiving O-heterobiotin until 1000 γ were included in the diet. It is interesting to note that this represents the same level at which a growth plateau was established. Similarly, no O-heterobiotin could be detected in muscle tissue until the 1000 γ level of the analog was reached.

Summary. d,l-O-Heterobiotin has biotin-like activity for the chick. Levels of 20 γ per 100 g of purified biotin-deficient basal ration give complete protection against dermatitis and some growth response. The growth activity of the oxygen analog varies inversely with the level fed and growth comparable to that on an adequate biotin diet was not obtained. d,l-O-Heterobiotin can be detected in the liver and muscle tissue of chicks fed high amounts of this compound.

15268

The Inactivation of Streptomycin and its Practical Applications.*†

WALTON B. GEIGER, SAMUEL R. GREEN, AND SELMAN A. WAKSMAN.
From the New Jersey Agricultural Experiment Station, New Brunswick, N. J.

Because of the rapidly accumulating information on the production, purification and practical utilization of streptomycin,[1] a knowledge of the effects of experimental conditions upon its activity is increasingly important. It is known, for example, that a weakly alkaline environment, namely pH 8.0 to 8.2, is favorable to the action of streptomycin and that acidic conditions are injurious.[2] Likewise, its activity is diminished by the presence of glucose and of phosphates. Cysteine and several ketone reagents also inactivate streptomycin.[3] Since factors of this sort can be of importance in choosing media for the testing of the potency of streptomycin, in developing tests for the sterility of streptomycin preparations, and in other practical applications, it was believed desirable to reinvestigate and extend some of the earlier observations. The presence of reducing agents, such as cysteine and glucose, among the substances that inactivate streptomycin, as well as the observation that anaerobic bacteria are somewhat resistant to its activity, has suggested that special attention be paid to these conditions, in an effort to learn whether their effects are of a fundamental

---

* Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Microbiology.

† Partly supported by a grant made by the Commonwealth Fund of New York.

[1] Waksman, S. A., and Schatz, A., *J. Am. Pharm. Assn.*, 1945, **34**, 273.

[2] Waksman, S. A., Bugie, E., and Schatz, A., *Proc. Staff Meet. Mayo Clinic*, 1944, **19**, 537.

[3] Denkewalter, R., Cook, M., and Tishler, M., *Science*, 1945, **101**, 12.

TABLE I.
Effect of Atmosphere Upon Action of Streptomycin.

Atmosphere	Activity in dilution units per ml against			
	E. coli	*A. aerogenes*	*Pr. vulgaris*	*S. marcescens*
Air	500	500	500	500
H_2	200	400	100	20
N_2	100	100	100	80
CO_2	20	20	30	20

TABLE II.
Effect of Bicarbonate and Acidity Upon Action of Streptomycin.

	Activity in units/ml against			
	E. coli	*A. aerogenes*	*S. aureus*	*B. subtilis*
Nutrient agar (pH 6.8)	100	100	100	1,000
Nutrient agar (pH 5.7)	10	10	5	100
Nutrient agar + 2% $NaHCO_3$	100	100	100	800

nature or are due to secondary factors such as fall in pH value.

Streptomycin is a rather stable compound. No biological agent or enzyme system has yet been found capable of destroying it. At 25°C, it is not affected appreciably between pH 2.0 and 9.0, but it is decomposed by excessive acidity (1 *N* HCl) and alkalinity (0.1 *N* NaOH). Hydrolysis by hydrochloric acid in methanol has been shown to result in the splitting of the molecule with the liberation of a base, streptidine, and a carbohydrate-like substance, streptobiosamine, neither of which possesses much, if any, antibiotic activity. The streptidine is believed to be attached to the streptobiosamine by a glucosidic linkage.[4]

Aerobic vs. Anaerobic Environment. In order to learn whether the greater resistance of anaerobes than of aerobes to streptomycin was due to the nature of the organisms or to the environment, a study was first made of the activity of streptomycin on several bacteria growing in different environments. Organisms that are facultative anaerobes, *i.e.*, that can grow either in air or with a limited supply of oxygen, were selected. A highly active streptomycin preparation containing 500 units/ml was used. Serial dilutions of the streptomycin solution were made in nutrient agar, and 4 series of plates were made up with this streptomycin-containing agar and streaked with the 4 test bacteria. One series of plates was incubated at 28°C under the usual aerobic conditions, and the other 3 series were incubated under anaerobic conditions in atmospheres of hydrogen, nitrogen, and carbon dioxide. The results presented in Table I show that streptomycin is about one-half as active under hydrogen as in air, considerably less active under nitrogen, and almost completely inactive under carbon dioxide.

Two supplementary tests were made in an effort to elucidate the effect of carbon dioxide. First, it was determined that the pH of nutrient agar was changed from 6.8 to 5.7 by storage for 16 hours under carbon dioxide. Tests showed that on agar adjusted to this pH, streptomycin had its activity diminished to about the same degree as by carbon dioxide. The effect of streptomycin was now tested on nutrient agar to which sodium bicarbonate had been added (Table II). No inactivation of streptomycin took place. One may, therefore, conclude that the effect of carbon dioxide is probably a result of the change in pH produced by it in the medium.

In order to determine whether the di-

---

[4] Brink, N. G., Kuehl, Jr., F. A., and Folkers, K., *Science*, 1945, **102**, 506.

TABLE III.
Changes in pH Value Produced by 4 Test Bacteria in Different Environments.

Atmosphere	Organism	pH*
Air	*E. coli*	7.50
	A. aerogenes	7.80
	Ser. marcescens	7.35
	Pr. vulgaris	7.13
Nitrogen	*E. coli*	7.25
	A. aerogenes	7.54
	Ser. marcescens	6.68
	Pr. vulgaris	6.69
Hydrogen	*E. coli*	6.63
	A. aerogenes	6.84
	Ser. marcescens	6.48
	Pr. vulgaris	6.43

* Original pH 7.0.

TABLE IV.
Effect of Sugars Upon Susceptibility of *B. subtilis* to Streptomycin.

Sugar added*	pH after growth	Cup method zone size in mm
None	7.1	19.3
Dextrose	6.3	10.1
Levulose	6.3	10.0
Sucrose	5.3	10.4
Mannose	7.1	18.4
Galactose	7.1	17.8
Arabinose	6.9	14.2
Maltose	7.0	18.5
Lactose	7.1	17.2
Mannitol	6.7	10.0

* 1% in nutrient agar.

minished activity of streptomycin in nitrogen or hydrogen could be a result of the production of acid by the test organisms under these conditions, a study was made of the changes in pH of the cultures. Flasks of nutrient broth were inoculated with the above 4 cultures and kept under air, hydrogen, and nitrogen. After incubation for 24 hours, pH determinations were made (Table III). The results suggest that the production of acid by the test bacteria under anaerobic conditions may be at least one factor in the diminished activity of streptomycin under these conditions.

Effect of Sugars. The action of streptomycin on *B. subtilis* is known to be diminished by glucose.[1] In order to obtain further information concerning this effect, the following 2 experiments were conducted. Since glucose is fermented by *B. subtilis* with the formation of acid, and since streptomycin is less active at low pH, it seemed likely that the action of glucose was largely a pH effect. A study was therefore made of the production of acid by *B. subtilis* from several sugars, as well as the effect of these sugars upon the activity of streptomycin. The sugars were incorporated in nutrient agar, which was inoculated with a spore suspension of *B. subtilis,* and plates were poured. Metal cups were placed upon the solidified agar plates and the streptomycin solution was added. The results presented in Table IV show that those sugars, namely dextrose, levulose, and sucrose which yield acid diminish most markedly the action of streptomycin. Mannose, galactose and lactose, from which little or no acid is produced, have little effect upon the activity of streptomycin.

When *E. coli* was used as the test organism (Table V) it was found that acid was produced by this particular strain from all the sugars, but in the smallest amount from lactose. Likewise all the sugars but lactose gave a diminution in the effectiveness of streptomycin against this organism.

That the inactivating effect of glucose upon streptomycin is due to the production of acid has been further confirmed by the following experiment: when the pH of the test medium was held at 8.0 by the addition of a glycine buffer, the addition of glucose did not result in the inactivation of streptomycin. Glycine alone had no effect upon the activity of streptomycin.

In view of observed effects of cysteine, the

TABLE V
Effect of Sugars Upon Susceptibility of *E. coli* to streptomycin.

Sugar added*	pH after growth	Cup method zone size in mm
None	6.5	17.5
Dextrose	5.1	15.0
Levulose	5.1	16.0
Sucrose	5.0	15.5
Mannose	5.0	15.0
Galactose	5.2	15.2
Arabinose	5.2	17.0
Maltose	5.3	16.8
Lactose	6.1	18.0
Mannitol	5.2	16.5

* 1% in streptomycin agar.

TABLE VI.
Effect of Cevitamic Acid Upon Action of Streptomycin.

Cevitamic acid	Activity, in dilution units per gram, against			
	E. coli	*A. aerogenes*	*S. aureus*	*B. subtilis*
0	100	100	50	800
0.4 mg/ml	10	10	5	100

TABLE VII.
Effect of Sulfhydryl Compounds on Action of Streptomycin.

Sulfhydryl compound	Zone size, mm	
	*B. subtilis**	*E. coli*†
None	18.0	27.0
Cysteine (0.02 *M*)	10.0	8.0
Thioglycolate (0.04 *M*)	17.0	25.5
Monothioglycol (0.04 *M*)	—	22.0
Glutathione	18.0	—

* Streptomycin concn. 25 units/ml medium, nutrient agar.

† Medium, streptomycin agar.

effect of another reducing agent—cevitamic acid—upon the activity of streptomycin was studied. The tests were made by the agar-streak method, using nutrient agar at pH 6.8 and nutrient agar containing 0.4 mg of cevitamic acid per ml and adjusted to pH 6.8. The results (Table VI) show that cevitamic acid greatly diminishes the effectiveness of streptomycin. Tests for the production of acid from cevitamic acid of this concentration showed that no detectible acid was produced by the test organisms. The cause of inhibition by the cevitamic acid is thus not immediately apparent.

Effect of Sulfhydryl Compounds. According to Denkewalter, Cook and Tishler[3] cysteine and β-mercaptoethylamine inhibited the action of streptomycin on *B. subtilis,* whereas thioglycolic acid was ineffective. A detailed study was made of this phenomenon, which fully confirmed their results and permitted the extension of their conclusions to include also the gram-negative *E. coli* (Table VII). It was also found that cysteine does not stimulate the production of acid by these organisms.

Thioglycolate agar (Brewer's) has been proposed as a medium for use in testing streptomycin for sterility. This medium contains not only thioglycolate, but also glucose, and is far richer in nitrogenous constituents than the usual nutrient agar. Accordingly, the effects of these constituents upon the activity of streptomycin were studied. The results of several experiments (Table VIII) show that the presence of 0.2% of sodium thioglycolate in nutrient agar did not inactivate streptomycin. Whereas the presence of 1.0% of glucose diminished the bacteriostatic effect of streptomycin. Glucose plus thioglycolate had no greater effect than glucose alone. With an increase in the concentration of the nitrogenous constituents in the medium, streptomycin was found generally less effective, but the absence of inactivation by thioglycolate, diminution of activity by glucose, and absence of synergistic action of the 2 substances were again observed.

Effect of Ketone Reagents. Brink, Kuehl and Folkers[3] have reported that "when streptomycin was treated with a variety of carbonyl group reagents, complete inactivation was observed." They mentioned particularly hydroxylamine and semicarbazide, and have isolated the oxime and semicarbazone. To confirm their results, solutions of streptomycin were mixed with the ketone reagents and the mixture was tested by the cup method (Table IX). Certain amino compounds which are not ketone reagents, such as ethylenediamine and methylamine, were without effect upon the activity of streptomycin.

Age of Culture. Streptomycin appears to have a more marked effect upon young actively growing cultures of bacteria than upon older cultures where the rate of growth has diminished. To confirm this, streptomycin was added to cultures of *E. coli* in nutrient broth which contained streptomycin, and after 8 hours' further incubation at 28°C, the number of viable cells were counted.

It was found that when streptomycin was

TABLE VIII.
Effect of Thioglycolate and Glucose Upon Action of Streptomycin.

Nutrient agar—agar-streak method

Substance added	Dilution units against		
	E. coli	*S. aureus*	*B. subtilis*
None	100	50	1000
Glucose*	30	20	200
Thioglycolate	100	50	1000
Glucose+ thioglycolate	30	20	30

Nutrient agar—cup method

	Zone of inhibition, mm	
	B. subtilis	*E. coli*
None	16.5	16.7
Glucose	10.0	14.5
Thioglycolate	15.3	16.2
Glucose+ thioglycolate	10.0	15.7

Enriched agar†—agar-streak method

	Dilution units against		
	E. coli	*S. aureus*	*B. subtilis*
None	10	50	300
Glucose	5	10	10
Thioglycolate	20	20	200
Glucose+ thioglycolate	10	10	50

* Glucose added in 1% concentration, and thioglycolate in 0.2%.

† Composition of enriched agar:

Beef extract	5 g
Peptone	20 g
NaCl	5 g
Agar	20 g
Dist. water	1000 ml

added to cultures from 0 to 3 hours old, a 75% reduction in growth resulted when the concentration of streptomycin was 2 units per ml and a 95% reduction in growth when the concentration was 5 units per ml. In cultures 24 to 48 hours old, the effect of streptomycin was less marked. A concentration of 2 units per ml caused a reduction in growth of 19%, and a concentration of 5 units per ml a reduction of 42%.

TABLE IX.
Effect of Ketone Reagents Upon Action of Streptomycin (25 units/ml).

Ketone reagent	Zone—size, ml	
	B. subtilis	*E. coli*
None	18	25
Hydroxylamine (.002 *M*)	10.5	8.0
Hydrazine (.002 *M*)	—	10.0
Semicarbazide (.002 *M*)	9.0	—
Phenylhydrazine (.002 *M*)	13.0	8.0
Methylphenylhydrazine (.001 *M*)	15.0	10.0

Discussion. The antibacterial activity of streptomycin can be largely or completely neutralized or antagonized by various chemical agents. These include glucose and certain other sugars, an anaerobic environment, certain sulfhydryl compounds, and ketone reagents. In some cases, as in the action of sugars or the anaerobic environment, the effect on streptomycin can be traced to the acidity produced under these particular conditions. However, in the effect of cysteine, cevitamic acid, and of ketone reagents the inhibition of streptomycin activity may be associated with the blocking of an active grouping in the molecule of the streptomycin. To explain the inactivation of the antibacterial properties of streptomycin by the blocking of a single group in its molecule cannot be attempted at present due to its rather complicated structure. Until the chemistry of streptomycin is more clearly elucidated, it is difficult to present a suitable theory that would account for the various effects of streptomycin inactivation.

15269

Blood Platelets in Heterophil Anaphylaxis.

A. Graña.* (Introduced by Hiram E. Essex).

From the Mayo Foundation, Rochester, Minn.

The serum of rabbits into which erythrocytes of sheep have been injected produces toxic symptoms and death when injected into guinea pigs. The reason for this was explained when Forssman[1] and others[2,3] announced the discovery and distribution of the so-called heterophil antigen. At this time it was found that the same antigenic substance which is present in erythrocytes of sheep is also present in the tissues of the guinea pig, dog and other species. Hence, the antibody produced in the rabbit in response to erythrocytes of sheep would also react with the antigen present in the tissues of the animal injected.

As the reactions exhibited by the animals following such injections of serum are almost identical with those of anaphylaxis, the phenomenon was designated heterophil anaphylaxis.

Later Taniguchi[4,5,6] observed that the lungs of guinea pigs killed by such injections of serum were markedly hemorrhagic and both lungs and trachea were filled with frothy fluid but were not markedly emphysematous as in true anaphylaxis. Furthermore Redfern[7] observed that no contraction is produced in the perfused uterine muscle following the addition of anti-Forssman serum. I have confirmed these facts shown by previous investigators. Moreover I found in collaboration with Recarte[8] that no increase of blood histamine was present in the acute shock induced in guinea pigs by the injection of anti-Forssman serum and no histamine was liberated when lungs of guinea pigs were perfused with such serums.

The experiments reported in the present paper were made with the aim of studying the modifications of the blood platelets in this particular type of shock. For this the volume of blood platelets was determined in guinea pigs and dogs before and after the injection of rabbit serum with high titer of antisheep hemolysin. At the same time the influence of injections of hepatic glycogen on the course of this type of shock was observed, since this substance in certain doses produces a temporary disappearance of the platelets of the blood.[9]

Methods. Thirteen unanesthetized male guinea pigs weighing from 500 to 1,030 grams were injected in the vein of the penis with vari-

---

*Guggenheim Fellow, from the Institute of Experimental Medicine, Montevideo, Uruguay.

[1] Forssman, J., and Hintze, Assar, *Biochem. Z.*, 1912, **44**, 336.

[2] Doerr, R., and Pick, R., *Biochem. Z.*, 1913, **50**, 129.

[3] Sachs, H., and Nathan, E., *Z. f. Immunitätsforchr. u. exp. Therap.*, 1913, **19**, 235.

[4] Taniguchi, Tenji, *J. Path. and Bact.*, 1920, **23**, 364.

[5] Taniguchi, Tenji, *J. Path. and Bact.*, 1921, **24**, 217.

[6] Taniguchi, Tenji, *J. Path. and Bact.*, 1922, **25**, 77.

[7] Redfern, W. W., *Am. J. Hyg.*, 1926, **6**, 276.

[8] Graña, A., and Recarte, P., *Rev. Soc. argent. de biol.*, 1945, **21**, 202.

[9] Rocha e Silva, M., Graña, A., and Porto, A., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 57.

TABLE I.
Blood Platelets and Leukocytes in Heterophil Anaphylaxis of the Guinea Pig.

Guinea pig No.	Weight, g	Antisheep rabbit serum, cc/100 g body wt.	Grade of shock	Blood platelets, vol. in 100 cc of blood		Leukocytes, thousands per cu mm of blood	
				Before inj.	After inj.	Before	After
1	500	0.10	Death in 2 min.	1.20	0	—	—
2	510	0.02	No shock	1.30	0	12.4	10.8
3	570	0.07	Death in 1 min.	1.20	0.05	14.8	5.6
4	535	0.01	No shock	0.85	0.85	12.3	11.8
5	530	0.02	” ”	0.85	0.20	14.4	5.0
6	500	0.02	Slight dyspnea	0.70	0	18.8	7.8
7	490	0.10	Death in 1 min.	0.80	0	—	—
8	510	0.015	No shock	0.60	0.05	12.0	11.2
9*	590	0.10	Death in 1 min.	0	0	—	—
10*	940	0.05	No shock	0.70	0	—	—
11*	1,030	0.10	Death in 1 min.	0.40	0	—	—
12*	1,000	0.15	Death in 2 min.	0	0	—	—
13*	900	0.10	Death in 1 min.	—	—	—	—

* The last 5 guinea pigs were injected with 1 g of glycogen in the vein of the penis before the injection of antisheep rabbit serum and the platelets were determined after the injection of glycogen and after the injection of serum.

able amounts of rabbit serum containing antisheep hemolysin. These serums were prepared by injecting erythrocytes of sheep into the rabbits, standard technics being followed. The hemolytic titer of the serum used in these experiments was 20,000 (that is, 0.00005 ml hemolyzed 0.5 ml of 2% sheep cells in the presence of 0.05 ml of pooled guinea pig serum as complement). Following intracutaneous injections of 0.10 ml of this serum into guinea pigs and dogs a notable hemorrhagic lesion appeared locally.

Five of the 13 guinea pigs used in these experiments were injected in the vein with 1 g of hepatic glycogen, dissolved in 5 ml of water, 3 minutes before the injection of the immune rabbit serum.

Four guinea pigs were injected with normal rabbit serum as controls and the volume of the platelets was determined before, and from 3 to 10 minutes after, the injection.

Different amounts of the immune rabbit serum were injected into 4 dogs anesthetized with pentobarbital sodium and with the femoral artery cannulated in order to determine the blood pressure. The volume of platelets was determined before and after the injection.

All specimens of blood were drawn directly from the heart of the guinea pigs and from the jugular vein of the dogs. In all the guinea pigs that manifested shock the blood samples were taken immediately following the appearance of symptoms. In animals without shock the samples were taken from 3 to 10 minutes after the injection of serum.

For the determinations of the platelets the Van Allen method[10] was used; in guinea pigs 2 ml of blood were taken in 3 ml of 3.8% solution of sodium citrate with 0.1% of formalin.

In several animals the modification of the leukocytes was studied and standard microscopic methods were used.

Results. From the data presented in Table I it may be observed that the immune rabbit serum employed in these experiments is highly toxic for the guinea pig, since 0.07 ml for each 100 g of body weight injected into the

---

[10] Van Allen, C. M., *J. Lab. and Clin. Med.*, 1926, **12**, 282.

TABLE II.
Blood Platelets in Guinea Pigs Injected with Normal Rabbit Serum.*

Guinea pig No.	Weight, g	Normal rabbit serum, cc/100 g body wt.	Blood platelets, volume in 100 cc of blood	
			Before	After
1	1,030	0.10	1.20	1.0
2	1,000	0.05	1.20	1.0
3	1,170	0.10	1.20	1.20
4	960	0.02	1.40	1.40

* None of these guinea pigs exhibited shock.

TABLE III.
Blood Platelets and Leukocytes in Heterophil Anaphylaxis of the Dog.

Dog No.	Weight, kg	Antisheep rabbit serum, cc/kg body wt.	Grade of shock	Blood platelets, volume in 100 cc of blood		Leukocytes, thousands per cu mm of blood	
				Before inj.	After inj.	Before	After
1	10	0.5	Death in 1 min.	0.60	0	—	—
2	10.3	0.20	Severe shock; death after 90 min.	0.90	0 (4 min.)		1.6
					0.2 (10 '')	17.0	2.6
					0.5 (90 '')		2.8
3	18	0.10	Slight shock; blood pressure decreased 15 mm Hg	0.60	0.17	7.2	0.6
4	6.8	0.10	Moderate shock; blood pressure decreased 90 mm Hg; recovery in 90 min.	0.55	0.05 (2 '')	3.0	0.4
					0.12 (30 '')		1.0
					0.35 (45 '')		1.4
					0.45 (5 hr.)		4.2

vein caused the death of the animal in one minute.

The lungs of guinea pigs and dogs that died in the course of these experiments showed deep macroscopic hemorrhagic lesions. The microscopic examination showed bronchial exudate in the terminal bronchi and striking adventitial hemorrhage around the larger vessels.

The volume of platelets was always drastically reduced after the injection of 0.02 ml or more per 100 g of body weight of this immune rabbit serum in spite of the fact that injections of small quantities of serum did not produce shock (Table I).

On the other hand, as shown in Table I, 4 of the last 5 guinea pigs which were injected intravenously with 1 g of glycogen, which eliminated all or nearly all of the blood platelets, were killed by an injection, 3 minutes later, of immune rabbit serum.

The experiments described show that in this special type of anaphylaxis the decrease of the blood platelets does not parallel the degree of shock as Kopeloff and Kopeloff[11] found in true anaphylaxis.

The reduction of blood platelets under these circumstances raises the question whether the phenomenon observed is specific or nonspecific. As may be seen from Table II, there was no significant change in volume of the blood platelets when normal rabbit serum was injected into guinea pigs.

The sharp fall of the blood platelets after the injection of the immune rabbit serum perhaps depends on a reaction produced in the blood between the antisheep hemolysin injected and the Forssman antigen present in the platelets of the guinea pig.

As is shown in Table III, the same drastic reduction of platelets was observed in dogs after the injection of antisheep hemolysin, although the shock was slight or moderate in dogs 3 and 4. Five hours after the injection of 0.10 ml of immune rabbit serum for each kilogram of body weight the volume of platelets reached approximately the normal

---

[11] Kopeloff, Nicholas, and Kopeloff, Lenore M., *J. Immunol.*, 1941, **40**, 471.

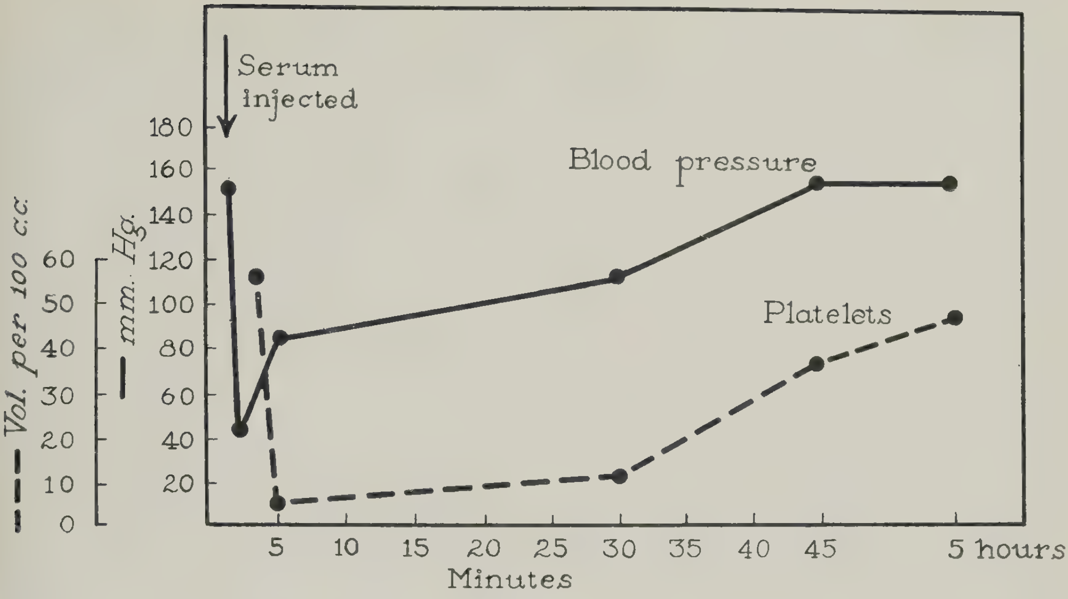


Fig. 1.

Platelets and blood pressure of a dog followed for 5 hours. As seen, 5 hours after the injection of 0.10 ml of immune rabbit serum for each kilogram of body weight the volume of platelets reached approximately the normal values.

values (Fig. 1). In addition to the reduction of platelets there was a drastic reduction of the number of leukocytes in the dog (Table III).

Summary. Serum of rabbits immune to sheep erythrocytes injected intravenously in appropriate doses in guinea pigs and dogs produced a drastic reduction of the concentration of blood platelets even though these quantities of serum provoked only moderate shock or no shock.

When the shock was deep, generally the platelets disappeared from the blood.

The injection of glycogen in doses that reduced the blood platelets in the guinea pig did not prevent the production of shock following the injection of the immune serum.

The experiments here presented show that there is not always a parallel relation between the decrease of blood platelets and the intensity of the pulmonary symptoms in the guinea pig or the decrease of arterial pressure of the dog in heterophil anaphylaxis.

15270

Subacute and Chronic Toxicities of Ascorbyl Palmitates.

O. GARTH FITZHUGH AND ARTHUR A. NELSON.

From the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

The fatty acid monoesters of l-ascorbic and d-isoascorbic acid prepared by Swern *et al.*[1] were suggested for use as antioxidants for fats and oils by Riemenschneider *et al.*[2] In cooperation* with these investigators we have made a study of the subacute and chronic oral toxicities of the palmitates of these acids.

---

[1] Swern, D., Stirton, A. J., Turer, J., and Wells, P. A., *Oil and Soap*, 1943, **20**, 224.

[2] Riemenschneider, R. W., Turer, J., Wells, P. A., and Ault, W. C., *Oil and Soap*, 1944, **21**, 47.

* The Eastern Regional Research Laboratory of

TABLE I.
Mean Gain in Weight of Male Rats Fed Diets Containing Palmitates for Six Months.

Substance	Dosage %	No. of Animals	Mean Gain Wt g	Standard Error of Mean g
l-ascorbyl	2	8	350.6	±17.1
palmitate	5	8	231.9	±22.3*
d-isoascorbyl	2	8	375.5	±14.3
palmitate	5	7	269.6	±15.1*
d-isoascorbic acid	1	10	405.3	±11.3
Control		9	394.7	±12.6

* $p = <.001$.

The results are reported in the following.

Experimental. For the subacute toxicity experiment 10 weanling male rats, selected at random with respect to litter mates from our colony of Osborne-Mendel strain, were placed on each of 6 diets. Ground commercial rat biscuits with 1% added cod liver oil served as the basic diet. The ascorbyl palmitates were mixed with the basic diet by means of a rotary batch mixer. Two groups of rats were placed on diets containing 2% and 5% concentrations of l-ascorbyl palmitate; two groups were placed on diets containing 2% and 5% d-isoascorbyl palmitate; one group was placed on a diet containing 1% d-isoascorbic acid and the sixth group received the control diet. Three rats were chosen from each of 6 litters and assigned to different diets as required for balanced incomplete blocks[3] of 3 with 6 treatments, where the blocks correspond to litters and treatments to diets. The design as a whole was replicated twice and thus a total of 60 rats was used. All animals were kept in individual cages in a room with controlled temperature and humidity and were given free access to their respective diets and water. Body weights and food consumption were determined at weekly intervals.

In the chronic toxicity study 6 groups of 10 rats each were selected and assigned to their respective diets similarly to those in the subacute experiment. In addition to a 5% lard sample, with or without added palmitate, the ingredients of the diets were casein 18%, dextrose 61%, brewer's yeast 5%, salt mixture (U.S.P. XII No. 2) 4%, corn oil 3%, whole liver powder 2%, and cod liver oil 2%. The ration for each of the 6 groups contained 5% of one of the following lard samples,[†] namely, heat-treated lard containing 1% or 5% l-ascorbyl palmitate, heat-treated lard containing 1% or 5% d-isoascorbyl palmitate, heat-treated lard without added palmitate, and fresh lard. All diets were refrigerated at —6.7°C.

Results, Subacute Toxicity. This experiment was terminated at 36 weeks when all surviving animals were sacrificed. The data on the gains in weight during the fast growing period of the first 26 weeks (Table I) show that the animals on the 5% concentrations of the two palmitates grew significantly ($p = <.001$) less than those on the control diet. At the 2% levels the differences between experimental and control animals were not statistically significant ($p = .05$, or less, is significant for this experiment). Although the gains in weight of the animals on the 1% d-isoascorbic acid were slightly greater than those of the controls, these differences were not significant. During the next 10 weeks, however, this group grew significantly faster than the controls. Because of this fact and also because all animals of the group were living, they were continued over a 2-year experiment; at the end of 2 years the surviving animals on the d-isoascorbic acid had no gross or microscopic pathological differ-

---

the U. S. Department of Agriculture furnished the palmitate monoesters and lard samples and partially defrayed the expenses of this study.

[3] Fisher, R. A., and Yates, F., *Statistical tables for biological, agricultural, and medical research*, Oliver and Boyd, Edinburgh, 1938.

[†] The palmitates were first incorporated in lard. The heat-treated lard samples with palmitates were prepared by heating fresh lard containing the palmitates at 100° C in a beaker with mechanical stirring and with free access to air for about 12 hours. The heat-treated lard without added palmitate was prepared by heating fresh lard at 100°C with washed air bubbling through the lard for 8 hours. Peroxide values showed that the heat-treated lard without the added palmitate was rancid.

ences from the control animals of other experiments and their mortality rate was what would be expected for control animals in our colony.

At 36 weeks, with the exception of the 1% d-isoascorbic acid group which showed no mortality, all groups of rats on the palmitate diets had fewer survivors than the group on the control diet. The figures for the percentage mortality were as follows: 5% d-isoascorbyl palmitate, 50%; 5% l-ascorbyl palmitate, 30%; 2% d-isoascorbyl palmitate and l-ascorbyl palmitate, 20% each; and the control 10%. The only group differing appreciably in mortality from the control, although not significantly with the small number of rats, was the 5% d-isoascorbyl group. Here there was a preponderance of deaths from acute pneumonia, but whether this should be attributed to the palmitates is doubtful.

All of the 60 rats started in the subacute series were autopsied, and 37 were microscopically sectioned. Lung, heart, liver, spleen, pancreas, stomach, small intestine, kidney, adrenal and testis were sectioned in all rats, while colon, lymph node, bone, bone marrow, thyroid and parathyroid were sectioned occasionally.

Two rats showed distinct lesions as a result of the palmitate feeding, and these were unusual. Both rats had been fed 5% l-ascorbyl palmitate for 36 weeks. Their urinary bladders were packed with numerous white mulberry stones up to 5 mm in diameter, one of which on crystallographic examination was found to consist of rods and needles of calcium oxalate monohydrate. Since oxalic acid has been reported[4] to be an oxidation product of ascorbic acid this would seem to be a reasonable source of the oxalate in the stones. In the feeding of over 50 substances to several thousand rats in 2-year chronic toxicity studies we have not observed spontaneous bladder stone formation. Stones have been produced with relative frequency by the feeding of glycols[5] and in one instance each by the feeding of barium chloride and saccharic acid. The last mentioned stone was composed of calcium oxalate, as were those caused by feeding glycols; the remaining stone was composed of barium sulphate in an organic matrix. In each rat with bladder stones one kidney and its ureter were markedly distended by the obstruction from the stones. Whitish material similar to the stones was seen in both kidney pelves of one rat. A small amount of colorless anisotropic crystalline calcareous material was seen in the parenchyma of one of the kidneys near the pelvis; this was the only kidney where such parenchymal crystalline material was seen. The kidneys in the 2 rats with urinary tract stones showed no inflammatory changes, although the bladders did show epithelial hyperplasia. Among the remaining 58 rats the surprising features were the almost total absence of calcareous material and of inflammatory changes within the kidneys and the lack of transitional examples in the gross appearance of the urinary tracts. Nor did the microscopic sections show any inflammatory changes or evidence of stone formation, with one exception, in a rat on 5% l-ascorbyl palmitate. There was a moderate degree of a focal, rather acute inflammatory process involving the interstitium and tubules of both cortex and medulla. Although this particular type of reaction has rarely been observed previously in our rats, it is not necessarily attributed to the palmitate, as unusual lesions do appear spontaneously in our rats from time to time.

Grossly, the viscera of the rats on the 5% palmitate diets, especially the l-ascorbyl palmitate, were slightly smaller than those of the controls and of the rats on 2% palmitate. Microscopically, except for the renal changes accompanying the 2 examples of stone formation and for the few spontaneous lesions that were observed, there was no outstanding difference among the 4 experimental groups and the control group. The amount of hemosiderin in the spleens of the group fed 5% palmitate was about half that in the remaining groups. The adrenals in all the experimental groups were slightly larger than those in the controls; however, there was no microscopic difference. The testes were undamaged, with rare exceptions.

---

[4] Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., *J. Biol. Chem.*, 1937, **117**, 237.

[5] Morris, H. J., Nelson, A. A., and Calvery, H. O., *J. Pharm. and Exp. Therap.*, 1942, **74**, 266.

Results, Chronic Toxicity. When the gains in weight were analyzed for the experimental and control animals for the first 24 weeks and for the first year, the differences were not statistically significant for any group. During the fast growing period individual growth curves of litter mates were enough alike to be considered identical. Growth curves of the experimental groups showed no significant deviation from the control, or from each other.

Each of the 4 rats which died during the first year was from a different group, therefore, their deaths were not related to treatment. During the second year deaths were distributed over the whole period and could not be attributed directly to the effect of the palmitates. At the termination of the experiment no group had a death rate greater than that of the control.

Histopathological study similar to that of the subacute toxicity series was made of the tissues of 36 of the 60 animals and showed no outstanding gross or microscopic difference between any of the experimental groups and the control group, nor were there any distinct lesions attributable to or prevented by the palmitates. The one group of rats which did differ slightly from all the others was that fed heat-treated lard without the added palmitates. In this group, the livers usually had a slight brown or chocolate tint rarely seen in the other groups. Microscopically, lymphocytic infiltration more frequently accompanied the small amount of focal renal tubular atrophy seen equally in all the groups, testicular atrophy was greater in degree than in all other groups, and lung involvement by chronic infectious processes and by lymphosarcoma was less frequent than in the rats whose diet included lard with added palmitates or unheated lard. However, an analysis of these data from the small number of animals in this experiment does not show a statistically significant difference.

Hematological studies made at intervals during the first year of the experiment showed no toxic effect on the blood from any of the chronic dosages of the palmitates.

Summary. When l-ascorbyl palmitate and d-isoascorbyl palmitate were fed subacutely for 9 months in concentrations of 2% and 5% of the diet, only the 5% levels were toxic. The growth rates of rats on the 5% levels were significantly retarded and bladder stones were produced in 2 animals on the 5% l-ascorbyl palmitate. d-Isoascorbic acid in a concentration of 1% of the diet had no deleterious effect. Chronic toxicity studies for 2 years indicate that l-ascorbyl and d-isoascorbyl palmitate are not toxic in concentrations of 0.25% (2500 p.p.m.), or less, of the total diet of rats. Heat-treating fresh lard containing the palmitates before adding it to the diet had no effect on toxicity.

15271

Use of Thermocouples for Determination of the Rectal Temperature of Rats.

David M. Tennent and Martha A. Meloy. (Introduced by H. Molitor).

From the Merck Institute for Therapeutic Research, Rahway, N. J.

When measurements of the rectal temperatures of rats were made in this laboratory with thermometers or with thermocouples while the animals were held in the investigator's hand, the readings were unstable and frequently changed by as much as one degree centigrade during the space of a few minutes. The results were also unsatisfactory when mechanical holders made of wire mesh were used, since the rats became unduly excited. To avoid these difficulties a method has been devised, using thermocouples, for taking the

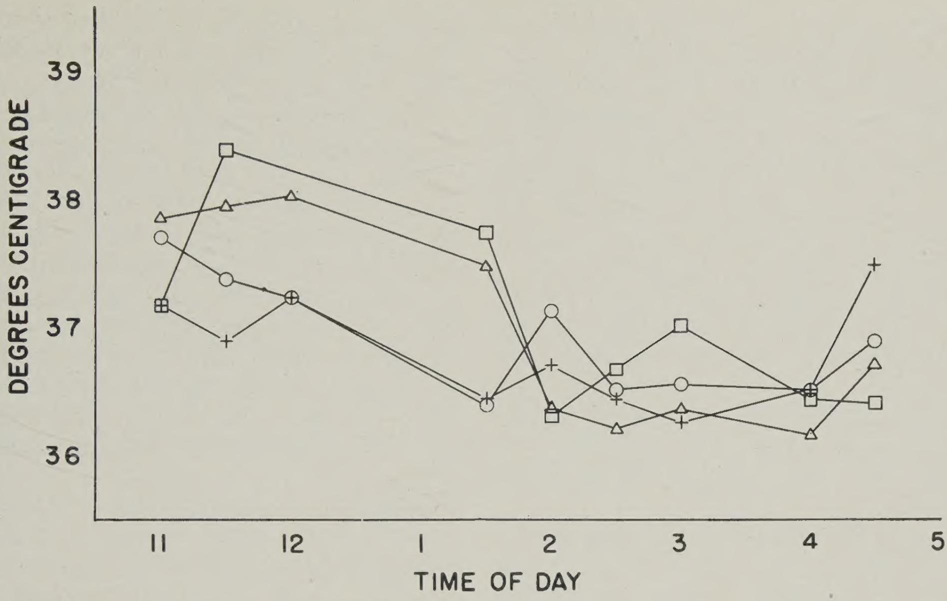


Fig. 1.
Body temperatures of 4 normal rats taken at intervals during the day.

temperatures of rats which are free in their cages and are neither excited nor subjected to a reduced rate of heat loss.

The thermocouples are made from 20 inch lengths of enamel and cotton covered iron and constantan wires, B & S gauge No. 30, which are cleaned and fused together at one end. The junction is then covered with a bead of hard solder about 2 mm in diameter which is smoothed with a file, and a 1½ inch length of rubber catheter tubing, size No. 8 French, is passed over the wires and pulled down tightly against the solder bead. A 10 mm length of rubber tubing, 7/32 inch outside diameter, ⅛ inch wall, fitted over the other end of the catheter tubing, serves to anchor the end of a 15 inch length of light steel helical pull spring, ¼ inch in diameter, through which the lead wires pass to the outside of the cage. The purpose of this spring is to protect these wires from attack by the rat.

It is important that proper attention be paid to the gauge of the thermocouple wires and to the size of the catheter tubing. If the assembled junction is too limber it will be forced out of the rectum during defecation, and if it is too stiff it will cause ulceration and perforation of the colon. The stiffness should be regulated by proper choice of the catheter tubing, and not by the gauge of the wires, which should be quite fine.

The thermocouples are attached outside the cage with silver solder to heavier wires of iron and of constantan which lead to a selector switch* making it possible to put a number of rats on test at the same time. The connections with the switch should be enclosed in a box to protect them from air currents.

The wire leading from the constantan side of the selector switch is attached to the constantan side of another thermocouple which is immersed in a reference bath of oil. This bath is mechanically stirred and electrically heated to a temperature of about 38°C, and it contains a calibrated thermometer. The iron sides of the reference junction and of the selector switch are attached through a double pole switch to the terminals of a sensitive galvanometer (5.50 u v per mm, internal resistance 2.1 ohms) and a precision wound variable resistance with a total value of 70 ohms is introduced into the lead from the selector switch so that the sensitivity of the galvanometer can be adjusted.

---

* Selector switches designed for use with thermocouples are available commercially and are preferable to the ones generally used in electronic equipment.

After calibration, this circuit will measure the differences between the temperatures of the rats and that of the reference bath to ±0.05°C. Each junction must be individually calibrated and will serve during daily use for about 10 days, failure being caused by rusting of the iron wire near the tip.

Specially designed potentiometers for use with iron-constantan thermocouples and containing automatic reference junction compensators are available commercially. Instruments of this type will afford greater convenience of measurement, but less sensitivity, than the circuit which has been described.

For use, the junctions are lubricated and inserted rectally to a depth equal to the length of the catheter tubing, and the end of the steel spring is firmly fastened with adhesive tape to the base of the rat's tail, which is first covered with a layer of gauze to minimize irritation. The adhesive tape is then covered with a piece of 40 mesh brass gauze (about 2¾ X ⅝ in. after the edges have been doubled over) which is held in place with 2 or 3 turns of wire.

After insertion of the thermocouples, the rats are returned to their cages, and the first readings may be taken about 30 minutes later. The junctions may be left in place for considerable periods of time. In the severest test to which the apparatus has been put, the temperatures of 9 rats were taken over 10 7-hour periods during 13 consecutive days. When the rats were sacrificed at the end of this time no lesions of the rectum or anus were found. Temperatures taken on a typical day are presented in Fig. 1.

Summary. 1. An apparatus for the measurement of the rectal temperatures of rats while they are free in their cages has been described. 2. An observed instability of readings which was presumably caused by reduction of heat loss or by excitation is avoided with this technic. 3. Typical results obtained at intervals during the day have been presented.

15272

Blood Concentration of P-chloro-xylenol in Man Following Parenteral, Percutaneous and Rectal Application.*

BERNHARD ZONDEK AND MICHAEL FINKELSTEIN.†

From the Obstetrical-Gynecological Department, Rothschild Hadassah University Hospital and the Hormone Research Laboratory, Hebrew University, Jerusalem, Palestine.

It has been shown[1,2] that halogenized phenols and especially p-chloro-xylenol (3.5-dimethyl, 4-chlorophenol, referred to subsequently as CX), and its derivatives exert a chemotherapeutic effect in man, in urogenital and general septic infections. Because of local irritation large amounts of these substances cannot be administered parenterally. We have, therefore, availed ourselves of experience gained in estrogenic hormone therapy[3] and applied CX percutaneously. Concurrently, CX is introduced rectally. In this paper it will be shown that free CX appears in blood of patients treated with CX by various routes of administration. CX was determined in blood according to Zondek *et al.*[4]

---

* Aided by a grant from the Rockefeller Foundation.

† We are deeply obliged to Drs. Bromberg, Brzezinski, Weber and Sulman for assistance rendered.

[1] Zondek, B., *Nature*, 1942, **149**, 334.

[2] Zondek, B., *J. Urology*, 1942, **48**, 747.

[3] Zondek, B., *Klin. Wschr.*, 1929, **48**, 2229; *Schwz. Med. Wschr.*, 1935, **65**, 1168.

[4] Zondek, B., Shapiro, B., and Hestrin, S., *Bioch. J.*, 1943, **37**, 589.

1. *CX in blood after intramuscular injection.* A 10% solution of CX in olive oil containing 2% anesthesin was employed. Other vehicles proved unsatisfactory. Doses of 10-20 cc (1 and 2 g CX respectively) were injected intragluteally into 12 healthy and diseased subjects. The blood was analyzed 2 to 6 times during the 48 hours following administration of the drug. In 3 cases no demonstrable amount of CX was found in the blood following administration of 1 g of CX, but in the other cases 1 mg% of CX was found 2-4 hours after the injection. The highest value found was 4 mg% 12 hours after injection in a case of hepatitis. With 2 g CX the blood content of CX was higher. In one case, 1.2 mg% was found 1 hour after injection, in 2 other cases 1.5 mg% and 3 mg% respectively 2 hours after injection. After 24 and 36 hours a concentration of 1 mg% was still demonstrated, whereas after 48 hours the blood contained no CX.

2. *Gastro-intestinal or rectal absorption of CX.* a. Stomach. CX on the tongue produces a numb sensation followed by burning. The oral administration of large quantities of this substance is therefore difficult. In one case it was possible to administer 10 g of CX dissolved in oil by this route without untoward after-effects. Blood analyses in this case gave the following results: after 1 hour—2 mg%; after 24 hours—1.4 mg%; after 48 hours—0. The blood concentration of CX after oral administration of 10 g does not exceed that obtained following intramuscular administration of 2 g of CX. We assume that this is due to removal of CX from the alimentary tract by the vena portae to the liver where by combination with glucuronate and sulfate it is rapidly inactivated.‡

b. Rectum. CX in 10% oil solution with 2% anesthesin can be administered rectally without injurious effect. Doses of 20 cc were administered once and twice daily, with a narrow catheter, backflow being prevented by closing the catheter with a clamp. Three patients were treated in this way. Following administration of 2.0, 2.5 and 4.0 g by this route, the following blood values were recorded: after 2 hours—traces, 1 mg% and 3 mg% respectively; after 24 hours—1 mg% and traces in the other cases; after 48 hours—traces in all 3 patients.

3. *Percutaneous administration.* Preliminary experiments indicated that large amounts of CX (10-30 g per day) can be administered percutaneously to man with no toxic effect. Eleven patients were treated percutaneously with 40% CX in alcohol-oil solution prepared by dissolving 100 g of CX in 100 cc ethanol and then adding an equal amount of olive oil gradually under continuous agitation. The results show that following percutaneous administration of a dose of 2 g, CX did not appear in demonstrable amount in the blood. After 5 g, traces were found, and after a dose of 8 g, 1 mg% CX was found after 3 hours, and 4 mg% after 24 hours. Following administration of larger doses (20 g), 4 mg% CX was demonstrable in the blood within one-half hour of the application and 1 mg% was still demonstrable in free form even after 72 hours.§

CX is thus resorbed by the skin, passes thence into the blood, and persists there for considerable periods of time. The prolonged presence of CX in the blood can probably be ascribed to the slow absorption of CX from the skin which thus acts as a depot.

4. *CX in the skin.* In conclusion it is of interest to note that skin treated with CX solutions or ointments remains sterile for 12 hours, a finding which in view of the known high disinfectant potency of CX is not unexpected. In performance of gynecological operations (Z.) the skin of the hypogastrium was vigorously anointed with 40% CX in oil-alcohol-solution 2-16 hours before operation. In one case 500 cm^2 of the skin was anointed

---

‡ Liver inactivates CX as will be reported elsewhere.

§ Unfortunately, we were unable to carry out similar experiments with CX-ointment, as our original American lanolin material was exhausted. In view of the greater bacteriostatic power of blood of patients who had received this CX-lanolin as compared with patients treated with CX-oil-alcohol solution it seems possible that the concentration of CX in the blood of these patients was correspondingly higher.

with 4.0 g CX 2 hours before operating. During operation 3 cm^2 of skin and subcutaneous tissue was removed and later analyzed. Without subcutaneous tissue it contained 2.5 mg CX, *i.e.* 10.4% of that applied to the area. In another case treated with 4.0 g CX on an area of 450 cm^2, 0.3 mg CX was found 15 hours after the inunction in 2.0 cm^2 skin, *i.e.* 1.7% of the CX applied. In the subcutaneous adipose tissue only 1-2 mg per g tissue were found. The findings show that CX penetrates into and through the skin.

Summary. The presence of free p-chloro-xylenol (CX) in the blood shows that this substance is resorbed after parenteral, oral, rectal, as well as percutaneous application. Only 1% of the amount of CX applied was detected in the blood. In percutaneous treatment, the skin acts as a depot from which CX is slowly resorbed into the blood, and as a result a prolonged circulation of CX in active form in the blood is obtained. The prolonged circulation of CX in the blood in active form following different routes of administration renders its chemotherapeutic efficacy in man comprehensible.